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(54) Title: IL-21 ANTAGONISTS

(57) Abstract: The polypeptides, and the polynucleotides encoding for them, described herein are IL-21 antagonists that bind with specificity and exhibit an EC<sub>50</sub> that is not detectable in receptor binding studies. These molecules have mutations in the D helix of the IL-21 molecule, and can be used to inhibit the activity of IL-21 with its cognate receptor.

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Description

## IL-21 ANTAGONISTS

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## BACKGROUND OF THE INVENTION

The interleukins are a family of cytokines that mediate immunological responses, including inflammation. The interleukins mediate a variety of inflammatory pathologies. Central to an immune response is the T cell, which produce many cytokines and adaptive immunity to antigens. Cytokines produced by the T cell have been classified as type 1 and type 2 (Kelso, A. Immun. Cell Biol. 76:300-317, 1998). Type 1 cytokines include IL-2, IFN- $\gamma$ , LT- $\alpha$ , and are involved in inflammatory responses, viral immunity, intracellular parasite immunity and allograft rejection. Type 2 cytokines include IL-4, IL-5, IL-6, IL-10 and IL-13, and are involved in humoral responses, helminth immunity and allergic response. Shared cytokines between Type 1 and 2 include IL-3, GM-CSF and TNF- $\alpha$ . There is some evidence to suggest that Type 1 and Type 2 producing T cell populations preferentially migrate into different types of inflamed tissue.

Mature T cells may be activated, i.e., by an antigen or other stimulus, to produce, for example, cytokines, biochemical signaling molecules, or receptors that further influence the fate of the T cell population. B cells can be activated via receptors on their cell surface including B cell receptor and other accessory molecules to perform accessory cell functions, such as production of cytokines.

The demonstrated *in vivo* activities of the cytokine family in inflammation and autoimmune disease illustrate the enormous clinical potential of, and need for cytokine antagonists. The present invention addresses these needs by providing antagonists of the IL-21 cytokine, as well as related compositions and methods.

The present invention provides such polypeptides for these and other uses that should be apparent to those skilled in the art from the teachings herein.

## SUMMARY OF THE INTENTION

In one aspect, the present invention provides an isolated polynucleotide molecule comprising a nucleotide sequence as shown in SEQ ID NO: 3 or SEQ ID NO: 5. In another aspect, the present invention provides an isolated polynucleotide

molecule encoding an IL-21 antagonist polypeptide comprising an amino acid sequence as shown as in SEQ ID NO: 4 or SEQ ID NO: 6. These sequences include mutations in the D helix of IL-21.

Embodiments of the present invention include In one embodiment of the present invention provides an antagonist molecule with a truncation of IL-21 polypeptide after residue 147 (Met) and wherein residue Gln<sub>145</sub> (SEQ ID NO: 2) was mutated to an Asp<sub>145</sub> (as shown in SEQ ID NO: 4). These mutations resulted in a protein with an IC<sub>50</sub> of 10 and EC<sub>50</sub> that was undetectable. The resulting polypeptide, was designated zalphall Ligand I156ST/Q153D (ST is a designation for truncation, in this case, at amino acid residue 156) shown to bind to the cognate receptor with specificity and without any detectable signaling.

In another embodiment, the present invention provides an antagonist molecule wherein Gln<sub>145</sub> (shown in SEQ ID NO: 2) has been mutated to Asp<sub>145</sub> (shown in SEQ ID NO: 6), and Ile<sub>148</sub> (shown in SEQ ID NO: 2) has been mutated to Asp<sub>148</sub> (shown in SEQ ID NO: 6). These mutations resulted in a protein with an IC<sub>50</sub> of 10 and an EC<sub>50</sub> that was undetectable. The resulting polypeptide, designated zalphall Ligand I156D/Q153D was shown to bind to the cognate receptor with specificity and without any detectable signaling.

One aspect of the present invention includes an isolated polypeptide comprising an amino acid sequence as shown in SEQ ID NO: 4 or SEQ ID NO: 6.

In another aspect, the present invention provides a fusion protein comprising at least two polypeptides, wherein at least one of the polypeptides comprises a polypeptide selected from the group consisting of an amino acid sequence as shown in SEQ ID NO:4 or SEQ ID NO:6, and a second polypeptide sequence.

An other aspect of the present invention provides an expression vector, comprising the isolated nucleic acid molecule of claim 1, a transcription promoter, and a transcription terminator, wherein the promoter is operably linked with the nucleic acid molecule, and wherein the nucleic acid molecule is operably linked with the transcription terminator.

Another aspect of the present invention provides a recombinant host cell comprising the expression vector of claim 5, wherein the host cell is selected from the group consisting of bacterium, yeast cell, fungal cell, insect cell, mammalian cell, and plant cell.

In another aspect, the present invention provides a method of producing a polypeptide, the method comprising the step of culturing recombinant host cells that comprise the expression vector of claim 5, and that produce the polypeptide.

## DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention in detail, it may be helpful to the understanding thereof to define the following terms:

The term "affinity tag" is used herein to denote a polypeptide segment  
5 that can be attached to a second polypeptide to provide for purification or detection of the second polypeptide or provide sites for attachment of the second polypeptide to a substrate. In principal, any peptide or protein for which an antibody or other specific binding agent is available can be used as an affinity tag. Affinity tags include a poly-histidine tract, protein A (Nilsson et al., EMBO J. 4:1075, 1985; Nilsson et al., Methods  
10 Enzymol. 198:3, 1991), glutathione S transferase (Smith and Johnson, Gene 67:31, 1988), Glu-Glu affinity tag (Grussenmeyer et al., Proc. Natl. Acad. Sci. USA 82:7952-4, 1985), substance P, Flag™ peptide (Hopp et al., Biotechnology 6:1204-10, 1988), streptavidin binding peptide, or other antigenic epitope or binding domain. See, in general, Ford et al., Protein Expression and Purification 2: 95-107, 1991. DNAs  
15 encoding affinity tags are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ).

The term "allelic variant" is used herein to denote any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within  
20 populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequence. The term allelic variant is also used herein to denote a protein encoded by an allelic variant of a gene.

The terms "amino-terminal" and "carboxyl-terminal" are used herein to denote positions within polypeptides. Where the context allows, these terms are used  
25 with reference to a particular sequence or portion of a polypeptide to denote proximity or relative position. For example, a certain sequence positioned carboxyl-terminal to a reference sequence within a polypeptide is located proximal to the carboxyl terminus of the reference sequence, but is not necessarily at the carboxyl terminus of the complete polypeptide.

The term "complement/anti-complement pair" denotes non-identical moieties that form a non-covalently associated, stable pair under appropriate conditions. For instance, biotin and avidin (or streptavidin) are prototypical members of a complement/anti-complement pair. Other exemplary complement/anti-complement  
30 pairs include receptor/ligand pairs, antibody/antigen (or hapten or epitope) pairs, sense/antisense polynucleotide pairs, and the like. Where subsequent dissociation of

the complement/anti-complement pair is desirable, the complement/anti-complement pair preferably has a binding affinity of  $<10^9 \text{ M}^{-1}$ .

The term "complements of a polynucleotide molecule" denotes a polynucleotide molecule having a complementary base sequence and reverse orientation as compared to a reference sequence. For example, the sequence 5' ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

The term "degenerate nucleotide sequence" denotes a sequence of nucleotides that includes one or more degenerate codons (as compared to a reference polynucleotide molecule that encodes a polypeptide). Degenerate codons contain different triplets of nucleotides, but encode the same amino acid residue (i.e., GAU and GAC triplets each encode Asp).

The term "expression vector" is used to denote a DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments include promoter and terminator sequences, and may also include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, etc. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both.

The term "isolated", when applied to a polynucleotide, denotes that the polynucleotide has been removed from its natural genetic milieu and is thus free of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes with which they are ordinarily associated, but may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, *Nature* 316:774-78, 1985).

An "isolated" polypeptide or protein is a polypeptide or protein that is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin. It is preferred to provide the polypeptides in a highly purified form, i.e. greater than 95% pure, more preferably greater than 99% pure. When used in this context, the term "isolated" does not exclude the presence of the same polypeptide in alternative physical forms, such as dimers or alternatively glycosylated or derivatized forms.

The term "neoplastic", when referring to cells, indicates cells undergoing new and abnormal proliferation, particularly in a tissue where the proliferation is uncontrolled and progressive, resulting in a neoplasm. The neoplastic cells can be either malignant, i.e. invasive and metastatic, or benign.

5           The term "operably linked", when referring to DNA segments, indicates that the segments are arranged so that they function in concert for their intended purposes, e.g., transcription initiates in the promoter and proceeds through the coding segment to the terminator.

10           The term "ortholog" denotes a polypeptide or protein obtained from one species that is the functional counterpart of a polypeptide or protein from a different species. Sequence differences among orthologs are the result of speciation.

"Paralogs" are distinct but structurally related proteins made by an organism. Paralogs are believed to arise through gene duplication. For example,  $\alpha$ -globin,  $\beta$ -globin, and myoglobin are paralogs of each other.

15           A "polynucleotide" is a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules. Sizes of polynucleotides are expressed as base pairs (abbreviated "bp"), nucleotides  
20 ("nt"), or kilobases ("kb"). Where the context allows, the latter two terms may describe polynucleotides that are single-stranded or double-stranded. When the term is applied to double-stranded molecules it is used to denote overall length and will be understood to be equivalent to the term "base pairs". It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide may differ slightly in length  
25 and that the ends thereof may be staggered as a result of enzymatic cleavage; thus all nucleotides within a double-stranded polynucleotide molecule may not be paired.

A "polypeptide" is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as "peptides".

30           The term "promoter" is used herein for its art-recognized meaning to denote a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes.

A "protein" is a macromolecule comprising one or more polypeptide  
35 chains. A protein may also comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents may be added to a protein by the cell in which the protein is produced, and will vary with the type of cell.

Proteins are defined herein in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless.

The term "receptor" denotes a cell-associated protein that binds to a bioactive molecule (i.e., a ligand) and mediates the effect of the ligand on the cell. Membrane-bound receptors are characterized by a multi-peptide structure comprising an extracellular ligand-binding domain and an intracellular effector domain that is typically involved in signal transduction. Binding of ligand to receptor results in a conformational change in the receptor that causes an interaction between the effector domain and other molecule(s) in the cell. This interaction in turn leads to an alteration in the metabolism of the cell. Metabolic events that are linked to receptor-ligand interactions include gene transcription, phosphorylation, dephosphorylation, increases in cyclic AMP production, mobilization of cellular calcium, mobilization of membrane lipids, cell adhesion, hydrolysis of inositol lipids and hydrolysis of phospholipids. In general, receptors can be membrane bound, cytosolic or nuclear; monomeric (e.g., thyroid stimulating hormone receptor, beta-adrenergic receptor) or multimeric (e.g., PDGF receptor, growth hormone receptor, IL-3 receptor, GM-CSF receptor, G-CSF receptor, erythropoietin receptor and IL-6 receptor).

The term "secretory signal sequence" denotes a DNA sequence that encodes a polypeptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger polypeptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

The term "splice variant" is used herein to denote alternative forms of RNA transcribed from a gene. Splice variation arises naturally through use of alternative splicing sites within a transcribed RNA molecule, or less commonly between separately transcribed RNA molecules, and may result in several mRNAs transcribed from the same gene. Splice variants may encode polypeptides having altered amino acid sequence. The term splice variant is also used herein to denote a protein encoded by a splice variant of an mRNA transcribed from a gene.

Molecular weights and lengths of polymers determined by imprecise analytical methods (e.g., gel electrophoresis) will be understood to be approximate values. When such a value is expressed as "about" X or "approximately" X, the stated value of X will be understood to be accurate to  $\pm 10\%$ .

All references cited herein are incorporated by reference in their entirety.

The present invention is based in part upon the discovery that certain mutations in the DNA encoding for interleukin 21 (IL-21) result in polypeptide

molecules that can bind the IL-21 receptor. In particular, mutations in the D helix IL-21 can be engineered and binding specificity retained for the IL-21 receptor. IL-21 was originally designated  $\alpha$ 11 ligand, and the receptor was originally designated  $\alpha$ 11. IL-21 is described in commonly owned U.S. Patent No. 6,307,024.

5 In general, cytokines are predicted to have a four- $\alpha$  helix structure, with helices A, C and D being most important in ligand-receptor interactions. In human IL-21 amino acid sequence as shown in SEQ ID NO:2, helix A is defined by amino acid residues 41-56; helix B by amino acid residues 69-84; helix C by amino acid residues 92-105; and helix D by amino acid residues 135-148. Structural analysis suggests that  
10 the A/B loop is long, the B/C loop is short and the C/D loop is parallel long. This loop structure results in an up-up-down-down helical organization. The cysteine residues are absolutely conserved between IL-21 and IL-15. The cysteine residues that are conserved between IL-15 and IL-21 correspond to amino acid residues 71, 78, 122 and 125 of SEQ ID NO: 2. Conservation of some of the cysteine residues is also found in  
15 IL-2, IL-4, GM-CSF and IL-21 corresponding to amino acid residues 78 and 125 of SEQ ID NO: 2. Consistent cysteine placement is further confirmation of the four-helical-bundle structure. Also highly conserved in the family comprising IL-15, IL-2, IL-4, GM-CSF and IL-21 is the Glu-Phe-Leu sequence as shown in SEQ ID NO: 2 at residues 136-138.

20 Analysis of IL-21 predicted that amino acid residues 44, 47 and 135 (as shown in SEQ ID NO: 2) played an important role in IL-21 binding to its cognate receptor. Moreover, the predicted amino acid sequence of murine IL-21 showed 57% identity to the predicted human protein. Based on comparison between sequences of human and murine IL-21 well-conserved residues were found in the regions predicted  
25 to encode  $\alpha$  helices A and D. The corresponding polynucleotides encoding the IL-21 polypeptide regions, domains, motifs, residues and sequences described herein are as shown in SEQ ID NO:1.

Detailed mutational analysis has been performed for IL-4 and IL-2, both of which are highly related to IL-21. Analysis of murine IL-2 (Zurawski et al., EMBO J. 12:5113-5119, 1993) shows residues in helices A and C are important for binding to IL-2R $\beta$ ; critical residues are Asp<sub>34</sub>, Asn<sub>99</sub>, and Asn<sub>103</sub>. Multiple residues within murine IL-2 loop A/B and helix B are important for IL-2R $\alpha$  binding, while only a single residue, Gln<sub>141</sub> in helix D, is vital for binding with IL-2R $\alpha$ . Similarly, helices A and C are sites of interaction between IL-4 and IL-4R $\alpha$  (the structurally similar to IL-2R $\alpha$ ),  
30 and residues within helix D are vital for IL-2R $\alpha$  interaction (Wang et al., Proc. Natl. Acad. Sci. USA 94:1657-1662, 1997; Kruse et al., EMBO J. 11:3237-3244, 1992). In



particular, the mutation Tyr<sub>124</sub> to Asp in human IL-4 creates an antagonist, which binds with IL-4R $\alpha$  but not IL-2R $\alpha$  and therefore cannot signal (Kruse et al. *ibid.* 1992).

While helix A is relatively well-conserved between human and murine IL-21, helix C is more divergent. While both species have predominant acidic amino acids in this region, the differences may account for species specificity in interaction between IL-21 and its "beta" type receptor,  $\alpha$ 11. Loop A/B and helix B of IL-21 are well-conserved between species; although no receptor subunit corresponding to IL-2R $\alpha$  has yet been identified, conservation through this region suggests that it is functionally significant. The D helices of human and murine IL-21 are also highly conserved. IL-21 receptor antagonists may be designed through mutations within IL-21 helix D. Any mutation which disrupts the IL-21 helical structure may abolish binding with its receptor and thus inhibit signaling.

In one embodiment of the present invention provides an antagonist molecule with a truncation of IL-21 polypeptide after residue 147 (Met) and wherein residue Gln<sub>145</sub> (SEQ ID NO: 2) was mutated to an Asp<sub>145</sub> (as shown in SEQ ID NO: 4). These mutations resulted in a protein with an IC<sub>50</sub> of 10 and EC<sub>50</sub> that was undetectable. The resulting polypeptide, designated  $\alpha$ 11 Ligand I156ST/Q153D (ST is a designation for truncation, in this case, at amino acid residue 156) shown to bind to the cognate receptor with specificity and without any detectable signaling.

In another embodiment, the present invention provides an antagonist molecule wherein Gln<sub>145</sub> (shown in SEQ ID NO: 2) has been mutated to Asp<sub>145</sub> (shown in SEQ ID NO: 6); and Ile<sub>148</sub> (shown in SEQ ID NO: 2) has been mutated to Asp<sub>148</sub> (shown in SEQ ID NO: 6). These mutations resulted in a protein with an IC<sub>50</sub> of 10 and an EC<sub>50</sub> that was undetectable. The resulting polypeptide, designated  $\alpha$ 11 Ligand I156D/Q153D was shown to bind to the cognate receptor with specificity and without any detectable signaling.

The functional domains of four-helical cytokines are determined on the basis of structural homology, irrespective of sequence identity, and can maintain functional integrity in a chimera (Kallen et al., *J. Biol. Chem.* 274:11859-11867, 1999). Therefore, the helical domains of IL-21 antagonists will be useful for preparing chimeric fusion molecules, particularly with other short-helix form cytokines to determine and modulate receptor binding specificity. Of particular interest are fusion proteins engineered with helix A, and fusion proteins that combine helical and loop domains from other short-form cytokines such as IL-2, IL-4, IL-15 and GM-CSF. The amino acid residues comprising helices A, B, C, and D, and loops A/B, B/C and C/D for IL-21, IL-2, IL-4, IL-15 and GM-CSF are shown in Table 1.

Table 1

	Helix A	A/B Loop	Helix B	B/C Loop	Helix C	C/D Loop	Helix D	
IL-21 residues	41-56	57-68	69-84	85-91	92-105	106- 134	135- 148	SEQ ID NO:2
IL-2 residues	36-46	47-52	53-75	76-86	87-99	100- 102	103- 121	SEQ ID NO: 7
IL-4 residues	29-43	44-64	65-83	84-94	95-118	119- 133	134- 151	SEQ ID NO: 8
IL-15 residues	45-68	69-83	84-101	102- 106	107- 119	120- 133	134- 160	SEQ ID NO: 9
GM- CSF residues	30-44	45-71	72-81	82-90	91-102	103- 119	120- 131	SEQ ID NO: 10

The IL-21 receptor is a member of the Class I cytokine receptor subfamily that includes the receptors for IL-2, IL-4, IL-7, IL-15, EPO, TPO, GM-CSF and G-CSF (for a review see, Cosman, "The Hematopoietin Receptor Superfamily" in Cytokine 5(2): 95-106, 1993). The IL-21 receptor is fully described in commonly-owned PCT Patent Application No. US99/22149. IL-21 receptor is expressed in lymph node, peripheral blood leukocytes (PBLs), spleen, bone marrow, and thymus. The tissue distribution of the receptor suggests that a target for the predicted IL-21 is hematopoietic lineage cells, in particular lymphoid progenitor cells and lymphoid cells. Other known four-helical-bundle cytokines that act on lymphoid cells include IL-2, IL-4, IL-7, and IL-15. For a review of four-helical-bundle cytokines, see, Nicola et al., Advances in Protein Chemistry 52:1-65, 1999 and Kelso, A., Immunol. Cell Biol. 76:300-317, 1998.

The present invention provides polynucleotide molecules, including DNA and RNA molecules, that encode the IL-21 antagonist polypeptides as disclosed herein. Those skilled in the art will readily recognize that, in view of the degeneracy of the genetic code, considerable sequence variation is possible among these polynucleotide molecules. SEQ ID NO:3 is a degenerate DNA sequence that encompasses all DNAs that encode the IL-21 polypeptide of SEQ ID NO:2. Those skilled in the art will recognize that the degenerate sequence of SEQ ID NO:3 also provides all RNA sequences encoding SEQ ID NO:2 by substituting U for T. Thus, IL-

21 polypeptide-encoding polynucleotides comprising nucleotide 1 or 97 to nucleotide 486 of SEQ ID NO:3 and their RNA equivalents are contemplated by the present invention. Table 2 sets forth the one-letter codes used within SEQ ID NO:3 to denote degenerate nucleotide positions. "Resolutions" are the nucleotides denoted by a code letter. "Complement" indicates the code for the complementary nucleotide(s). For example, the code Y denotes either C or T, and its complement R denotes A or G, with A being complementary to T, and G being complementary to C.

TABLE 2

Nucleotide	Resolution	Complement	Resolution
A	A	T	T
C	C	G	G
G	G	C	C
T	T	A	A
R	A G	Y	C T
Y	C T	R	A G
M	A C	K	G T
K	G T	M	A C
S	C G	S	C G
W	A T	W	A T
H	A C T	D	A G T
B	C G T	V	A C G
V	A C G	B	C G T
D	A G T	H	A C T
N	A C G T	N	A C G T

The degenerate codons used in SEQ ID NO:3, encompassing all possible codons for a given amino acid, are set forth in Table 3.

TABLE 3

Amino Acid	One Letter Code	Codons	Degenerate Codon
Cys	C	TGC TGT	TGY
Ser	S	AGC AGT TCA TCC TCG TCT	WSN
Thr	T	ACA ACC ACG ACT	ACN
Pro	P	CCA CCC CCG CCT	CCN
Ala	A	GCA GCC GCG GCT	GCN
Gly	G	GGA GGC GGG GGT	GGN
Asn	N	AAC AAT	AAY
Asp	D	GAC GAT	GAY
Glu	E	GAA GAG	GAR
Gln	Q	CAA CAG	CAR
His	H	CAC CAT	CAY
Arg	R	AGA AGG CGA CGC CGG CGT	MGN
Lys	K	AAA AAG	AAR
Met	M	ATG	ATG
Ile	I	ATA ATC ATT	ATH
Leu	L	CTA CTC CTG CTT TTA TTG	YTN
Val	V	GTA GTC GTG GTT	GTN
Phe	F	TTC TTT	TTY
Tyr	Y	TAC TAT	TAY
Trp	W	TGG	TGG
Ter	.	TAA TAG TGA	TRR
Asn Asp	B		RAY
Glu Gln	Z		SAR
Any	X		NNN

One of ordinary skill in the art will appreciate that some ambiguity is introduced in determining a degenerate codon, representative of all possible codons encoding each amino acid. For example, the degenerate codon for serine (WSN) can, in some circumstances, encode arginine (AGR), and the degenerate codon for arginine (MGN) can, in some circumstances, encode serine (AGY). A similar relationship exists between codons encoding phenylalanine and leucine. Thus, some polynucleotides encompassed by the degenerate sequence may encode variant amino acid sequences, but one of ordinary skill in the art can easily identify such variant sequences by reference to the amino acid sequence of SEQ ID NO:2. Variant sequences can be readily tested for functionality as described herein.

Using isolated polynucleotides of the present invention that include DNA and RNA, the native sequence of IL-21 is isolated for a template to identify mutants. Methods for preparing DNA and RNA are well known in the art.

The present invention also provides IL-21 antagonist polypeptides that have a substantially similar sequence identity to the polypeptides of SEQ ID NOS:4 or 6. The term "substantially similar sequence identity" is used herein to denote polypeptides comprising at least greater than 95% sequence identity to the sequences shown in SEQ ID NOS:4 or 6. The present invention further includes nucleic acid molecules that encode such polypeptides. Methods for determining percent identity are described below.

The present invention also contemplates variant IL-21 nucleic acid molecules that can be identified using two criteria: a determination of the similarity between the encoded polypeptide with the amino acid sequence of SEQ ID NOS:4 or 6, and/or a hybridization assay. Such IL-21 variants include nucleic acid molecules: (1) that hybridize with a nucleic acid molecule having the nucleotide sequence of SEQ ID NOS:3 or 5 (or their complements) under stringent washing conditions, in which the wash stringency is equivalent to 0.5x - 2x SSC with 0.1% SDS at 55 - 65°C; or (2) that encode a polypeptide having at least greater than 95% sequence identity to the amino acid sequence of SEQ ID NOS:4 or 6. Alternatively, IL-21 antagonists can be characterized as nucleic acid molecules: (1) that hybridize with a nucleic acid molecule having the nucleotide sequence of SEQ ID NOS:3 or 5 (or their complements) under highly stringent washing conditions, in which the wash stringency is equivalent to 0.1x - 0.2x SSC with 0.1% SDS at 50 - 65°C; and (2) that encode a polypeptide having at least greater than 95% sequence identity to the amino acid sequence of SEQ ID NOS:4 or 6.

Percent sequence identity is determined by conventional methods. See, for example, Altschul et al., Bull. Math. Bio. 48:603 (1986), and Henikoff and

Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1992). Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "BLOSUM62" scoring matrix of Henikoff and Henikoff (*ibid.*) as shown in Table 4 (amino acids are indicated by the standard one-letter codes).

Total number of identical matches

\_\_\_\_\_ x 100

[length of the longer sequence plus the  
number of gaps introduced into the longer  
sequence in order to align the two sequences]

Table 4

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	4																			
R	-1	5																		
N	-2	0	6																	
D	-2	-2	1	6																
C	0	-3	-3	-3	9															
Q	-1	1	0	0	-3	5														
E	-1	0	0	2	-4	2	5													
G	0	-2	0	-1	-3	-2	-2	6												
H	-2	0	1	-1	-3	0	0	-2	8											
I	-1	-3	-3	-3	-1	-3	-3	-4	-3	4										
L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4									
K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5								
M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5							
F	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6						
P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7					
S	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4				
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5			
W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11		
Y	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7	
V	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4

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Those skilled in the art appreciate that there are many established algorithms available to align two amino acid sequences. The "FASTA" similarity search algorithm of Pearson and Lipman is a suitable protein alignment method for examining the level of identity shared by an amino acid sequence disclosed herein and the amino acid sequence of a putative variant IL-21. The FASTA algorithm is described by Pearson and Lipman, Proc. Nat'l Acad. Sci. USA 85:2444 (1988), and by Pearson, Meth. Enzymol. 183:63 (1990).

Briefly, FASTA first characterizes sequence similarity by identifying regions shared by the query sequence (*e.g.*, SEQ ID NOS:4 or 6) and a test sequence that have either the highest density of identities (if the ktup variable is 1) or pairs of identities (if ktup=2), without considering conservative amino acid substitutions, insertions, or deletions. The ten regions with the highest density of identities are then rescored by comparing the similarity of all paired amino acids using an amino acid substitution matrix, and the ends of the regions are "trimmed" to include only those residues that contribute to the highest score. If there are several regions with scores greater than the "cutoff" value (calculated by a predetermined formula based upon the length of the sequence and the ktup value), then the trimmed initial regions are examined to determine whether the regions can be joined to form an approximate alignment with gaps. Finally, the highest scoring regions of the two amino acid sequences are aligned using a modification of the Needleman-Wunsch-Sellers algorithm (Needleman and Wunsch, J. Mol. Biol. 48:444 (1970); Sellers, SIAM J. Appl. Math. 26:787 (1974)), which allows for amino acid insertions and deletions. Preferred parameters for FASTA analysis are: ktup=1, gap opening penalty=10, gap extension penalty=1, and substitution matrix=BLOSUM62. These parameters can be introduced into a FASTA program by modifying the scoring matrix file ("SMATRIX"), as explained in Appendix 2 of Pearson, Meth. Enzymol. 183:63 (1990).

FASTA can also be used to determine the sequence identity of nucleic acid molecules using a ratio as disclosed above. For nucleotide sequence comparisons, the ktup value can range between one to six, preferably from three to six, most preferably three, with other parameters set as default.

Variant IL-21 polypeptides or polypeptides with substantially similar sequence identity are characterized as having one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions (see Table 5) and other substitutions that do not significantly affect the folding or activity of the polypeptide; small deletions, typically of one to about 30 amino acids; and amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25



residues, or an affinity tag. The present invention thus includes polypeptides of from about 108 to 216 amino acid residues that comprise a sequence that is at least 95% or more identical to the corresponding region of SEQ ID NOS:4 or 6. Polypeptides comprising affinity tags can further comprise a proteolytic cleavage site between the IL-21 polypeptide and the affinity tag. Preferred such sites include thrombin cleavage sites and factor Xa cleavage sites.

Table 5

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Conservative amino acid substitutions

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Basic:	arginine
	lysine
	histidine
Acidic:	glutamic acid
	aspartic acid
Polar:	glutamine
	asparagine
Hydrophobic:	leucine
	isoleucine
	valine
Aromatic:	phenylalanine
	tryptophan
	tyrosine
Small:	glycine
	alanine
	serine
	threonine
	methionine

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Determination of amino acid residues that comprise regions or domains that are critical to maintaining structural integrity can be determined. Within these

regions one can determine specific residues that will be more or less tolerant of change and maintain the overall tertiary structure of the molecule. Methods for analyzing sequence structure include, but are not limited to alignment of multiple sequences with high amino acid or nucleotide identity, secondary structure propensities, binary patterns, complementary packing and buried polar interactions (Barton, Current Opin. Struct. Biol. 5:372-376, 1995 and Cordes et al., Current Opin. Struct. Biol. 6:3-10, 1996). In general, when designing modifications to molecules or identifying specific fragments determination of structure will be accompanied by evaluating activity of modified molecules. The effects of amino acid sequence changes can be predicted by, for example, computer modeling as disclosed above or determined by analysis of crystal structure (see, e.g., Laphorn et al., Nat. Struct. Biol. 2:266-268, 1995). Other techniques that are well known in the art compare folding of a variant protein to a standard molecule (e.g., the native protein). For example, comparison of the cysteine pattern in a variant and standard molecules can be made. Mass spectrometry and chemical modification using reduction and alkylation provide methods for determining cysteine residues which are associated with disulfide bonds or are free of such associations (Bean et al., Anal. Biochem. 201:216-226, 1992; Gray, Protein Sci. 2:1732-1748, 1993; and Patterson et al., Anal. Chem. 66:3727-3732, 1994). It is generally believed that if a modified molecule does not have the same cysteine pattern as the standard molecule folding would be affected. Another well known and accepted method for measuring folding is circular dichroism (CD). Measuring and comparing the CD spectra generated by a modified molecule and standard molecule is routine (Johnson, Proteins 7:205-214, 1990). Crystallography is another well known method for analyzing folding and structure. Nuclear magnetic resonance (NMR), digestive peptide mapping and epitope mapping are also known methods for analyzing folding and structural similarities between proteins and polypeptides (Schaanan et al., Science 257:961-964, 1992).

Routine deletion analyses of nucleic acid molecules can be performed to obtain functional fragments of a nucleic acid molecule that encodes a IL-21 polypeptide. As an illustration, DNA molecules having the nucleotide sequence of SEQ ID NOS:3 or 5 or fragments thereof, can be digested with *Bal31* nuclease to obtain a series of nested deletions. These DNA fragments are then inserted into expression vectors in proper reading frame, and the expressed polypeptides are isolated and tested for IL-21 activity, or for the ability to bind anti-IL-21 antibodies or IL-21 receptor. One alternative to exonuclease digestion is to use oligonucleotide-directed mutagenesis to introduce deletions or stop codons to specify production of a desired IL-21 fragment.

Alternatively, particular fragments of a IL-21 gene can be synthesized using the polymerase chain reaction.

Standard methods for identifying functional domains are well-known to those of skill in the art. For example, studies on the truncation at either or both termini of interferons have been summarized by Horisberger and Di Marco, Pharmac. Ther. 66:507 (1995). Moreover, standard techniques for functional analysis of proteins are described by, for example, Treuter et al., Molec. Gen. Genet. 240:113 (1993); Content et al., "Expression and preliminary deletion analysis of the 42 kDa 2-5A synthetase induced by human interferon," in Biological Interferon Systems, Proceedings of ISIR-TNO Meeting on Interferon Systems, Cantell (ed.), pages 65-72 (Nijhoff 1987); Herschman, "The EGF Receptor," in Control of Animal Cell Proliferation 1, Boynton et al., (eds.) pages 169-199 (Academic Press 1985); Coumailleau et al., J. Biol. Chem. 270:29270 (1995); Fukunaga et al., J. Biol. Chem. 270:25291 (1995); Yamaguchi et al., Biochem. Pharmacol. 50:1295 (1995); and Meisel et al., Plant Molec. Biol. 30:1 (1996).

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer (Science 241:53 (1988)) or Bowie and Sauer (Proc. Nat'l Acad. Sci. USA 86:2152 (1989)). Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display (e.g., Lowman *et al.*, Biochem. 30:10832 (1991), Ladner et al., U.S. Patent No. 5,223,409, Huse, international publication No. WO 92/06204), and region-directed mutagenesis (Derbyshire et al., Gene 46:145 (1986), and Ner et al., DNA 7:127, (1988)).

Variants of the disclosed IL-21 nucleotide and polypeptide sequences can also be generated through DNA shuffling as disclosed by Stemmer, Nature 370:389 (1994), Stemmer, Proc. Natl Acad. Sci. USA 91:10747 (1994), and international publication No. WO 97/20078. Briefly, variant DNA molecules are generated by *in vitro* homologous recombination by random fragmentation of a parent DNA followed by reassembly using PCR, resulting in randomly introduced point mutations. This technique can be modified by using a family of parent DNA molecules, such as allelic variants or DNA molecules from different species, to introduce additional variability into the process. Selection or screening for the desired activity, followed by additional iterations of mutagenesis and assay provides for rapid "evolution" of sequences by selecting for desirable mutations while simultaneously selecting against detrimental changes.

Mutagenesis methods as disclosed herein can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides in host cells. Mutagenized DNA molecules that encode biologically active polypeptides, or polypeptides that bind with anti-IL-21 antibodies or soluble IL-21 receptor, can be recovered from the host cells and rapidly sequenced using modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

In addition, the proteins of the present invention (or polypeptide fragments thereof) can be joined to other bioactive molecules, particularly other cytokines, to provide multi-functional molecules. For example, one or more helices from IL-21 can be joined to other cytokines to enhance their biological properties or efficiency of production.

The present invention thus provides a series of novel, hybrid molecules in which a segment comprising one or more of the helices of IL-21 is fused to another polypeptide. Fusion is preferably done by splicing at the DNA level to allow expression of chimeric molecules in recombinant production systems. The resultant molecules are then assayed for such properties as improved solubility, improved stability, prolonged clearance half-life, improved expression and secretion levels, and pharmacodynamics. Such hybrid molecules may further comprise additional amino acid residues (e.g. a polypeptide linker) between the component proteins or polypeptides.

Non-naturally occurring amino acids include, without limitation, *trans*-3-methylproline, 2,4-methanoproline, *cis*-4-hydroxyproline, *trans*-4-hydroxyproline, *N*-methylglycine, *allo*-threonine, methylthreonine, hydroxyethylcysteine, hydroxyethylhomocysteine, nitroglutamine, homoglutamine, pipecolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, 3,3-dimethylproline, *tert*-leucine, norvaline, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, and 4-fluorophenylalanine. Several methods are known in the art for incorporating non-naturally occurring amino acid residues into proteins. For example, an *in vitro* system can be employed wherein nonsense mutations are suppressed using chemically aminoacylated suppressor tRNAs. Methods for synthesizing amino acids and aminoacylating tRNA are known in the art. Transcription and translation of plasmids containing nonsense mutations is typically carried out in a cell-free system comprising an *E. coli* S30 extract and commercially available enzymes and other reagents. Proteins are purified by chromatography. See, for example, Robertson et al., *J. Am. Chem. Soc.* **113**:2722 (1991), Ellman et al., *Methods Enzymol.* **202**:301 (1991), Chung et al.,

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Science 259:806 (1993), and Chung et al., Proc. Nat'l Acad. Sci. USA 90:10145 (1993).

In a second method, translation is carried out in *Xenopus* oocytes by microinjection of mutated mRNA and chemically aminoacylated suppressor tRNAs (Turcatti et al., J. Biol. Chem. 271:19991 (1996)). Within a third method, *E. coli* cells are cultured in the absence of a natural amino acid that is to be replaced (e.g., phenylalanine) and in the presence of the desired non-naturally occurring amino acid(s) (e.g., 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, or 4-fluorophenylalanine). The non-naturally occurring amino acid is incorporated into the protein in place of its natural counterpart. See, Koide et al., Biochem. 33:7470 (1994). Naturally occurring amino acid residues can be converted to non-naturally occurring species by *in vitro* chemical modification. Chemical modification can be combined with site-directed mutagenesis to further expand the range of substitutions (Wynn and Richards, Protein Sci. 2:395 (1993). It may be advantageous to stabilize IL-21 to extend the half-life of the molecule, particularly for extending metabolic persistence in an active state. To achieve extended half-life, IL-21 molecules can be chemically modified using methods described herein. PEGylation is one method commonly used that has been demonstrated to increase plasma half-life, increased solubility, and decreased antigenicity and immunogenicity (Nucci et al., Advanced Drug Delivery Reviews 6:133-155, 1991 and Lu et al., Int. J. Peptide Protein Res. 43:127-138, 1994).

A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, non-naturally occurring amino acids, and unnatural amino acids may be substituted for IL-21 amino acid residues.

The present invention also provides polypeptide fragments or peptides comprising an epitope-bearing portion of a IL-21 polypeptide described herein. Such fragments or peptides may comprise an "immunogenic epitope," which is a part of a protein that elicits an antibody response when the entire protein is used as an immunogen. Immunogenic epitope-bearing peptides can be identified using standard methods (see, for example, Geysen et al., Proc. Nat'l Acad. Sci. USA 81:3998 (1983)).

Regardless of the particular nucleotide sequence of a IL-21 antagonist polynucleotide, the polynucleotide encodes a polypeptide that is characterized by its ability to induce or inhibit specialized cell functions, or by the ability to bind specifically to an anti-IL-21 antibody or IL-21 receptor. More specifically, an IL-21 antagonist will bind its cognate receptor with at least an  $IC_{50}$  of 100 nM and exhibit an  $EC_{50}$  of 100 nM or greater.

For any IL-21 polypeptide, including variants and fusion proteins, one of ordinary skill in the art can readily generate a fully degenerate polynucleotide sequence encoding that variant using the information set forth in Tables 1 and 2 above.

The present invention further provides a variety of other polypeptide fusions (and related multimeric proteins comprising one or more polypeptide fusions). For example, a IL-21 polypeptide can be prepared as a fusion to a dimerizing protein as disclosed in U.S. Patents Nos. 5,155,027 and 5,567,584. Preferred dimerizing proteins in this regard include immunoglobulin constant region domains. Immunoglobulin- IL-21 polypeptide fusions can be expressed in genetically engineered cells (to produce a variety of multimeric IL-21 analogs). Auxiliary domains can be fused to IL-21 polypeptides to target them to specific cells, tissues, or macromolecules. For example, a IL-21 polypeptide or protein could be targeted to a predetermined cell type by fusing a IL-21 polypeptide to a ligand that specifically binds to a receptor on the surface of that target cell. In this way, polypeptides and proteins can be targeted for therapeutic or diagnostic purposes. A IL-21 polypeptide can be fused to two or more moieties, such as an affinity tag for purification and a targeting domain. Polypeptide fusions can also comprise one or more cleavage sites, particularly between domains. See, Tuan et al., Connective Tissue Research 34:1-9, 1996.

Using the methods discussed herein, one of ordinary skill in the art can identify and/or prepare a variety of polypeptides that have substantially similar sequence identity to residues 1-162 or 33-162 of SEQ ID NOS: 4 or 6, wherein such polypeptides or fusions bind the  $\alpha$ 11 receptor or IL-21 antibodies with an  $IC_{50}$  of 100 nM or less, and exhibit an  $EC_{50}$  of 100 nM or greater.

The IL-21 polypeptides of the present invention, including full-length polypeptides, functional fragments, and fusion polypeptides, can be produced in genetically engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells. Eukaryotic cells, particularly cultured cells of multicellular organisms, are preferred. Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, and Ausubel et al., eds., Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987.

In general, a DNA sequence encoding a IL-21 polypeptide is operably linked to other genetic elements required for its expression, generally including a transcription promoter and terminator, within an expression vector. The vector will

also commonly contain one or more selectable markers and one or more origins of replication, although those skilled in the art will recognize that within certain systems selectable markers may be provided on separate vectors, and replication of the exogenous DNA may be provided by integration into the host cell genome. Selection  
5 of promoters, terminators, selectable markers, vectors and other elements is a matter of routine design within the level of ordinary skill in the art. Many such elements are described in the literature and are available through commercial suppliers.

To direct a IL-21 polypeptide into the secretory pathway of a host cell, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre  
10 sequence) is provided in the expression vector. The secretory signal sequence may be that of  $\alpha$ 11 Ligand, or may be derived from another secreted protein (e.g., t-PA) or synthesized *de novo*. The secretory signal sequence is operably linked to the IL-21 DNA sequence, i.e., the two sequences are joined in the correct reading frame and positioned to direct the newly synthesized polypeptide into the secretory pathway of the  
15 host cell. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the polypeptide of interest, although certain secretory signal sequences may be positioned elsewhere in the DNA sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830).

Alternatively, the secretory signal sequence contained in the  
20 polypeptides of the present invention is used to direct other polypeptides into the secretory pathway. The present invention provides for such fusion polypeptides. A signal fusion polypeptide can be made wherein a secretory signal sequence derived from amino acid residue 1-31 of SEQ ID NO:2 is operably linked to a DNA sequence encoding another polypeptide using methods known in the art and disclosed  
25 herein. The secretory signal sequence contained in the fusion polypeptides of the present invention is preferably fused amino-terminally to an additional peptide to direct the additional peptide into the secretory pathway. Such constructs have numerous applications known in the art. For example, these novel secretory signal sequence fusion constructs can direct the secretion of an active component of a normally non-  
30 secreted protein. Such fusions may be used *in vivo* or *in vitro* to direct peptides through the secretory pathway.

Cultured mammalian cells are suitable hosts within the present invention. Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection (Wigler et al., Cell 14:725, 1978; Corsaro and  
35 Pearson, Somatic Cell Genetics 7:603, 1981; Graham and Van der Eb, Virology 52:456, 1973), electroporation (Neumann et al., EMBO J. 1:841-5, 1982), DEAE-dextran mediated transfection (Ausubel et al., ibid.), and liposome-mediated transfection



(Hawley-Nelson et al., *Focus* 15:73, 1993; Ciccarone et al., *Focus* 15:80, 1993, and viral vectors (Miller and Rosman, *BioTechniques* 7:980-90, 1989; Wang and Finer, *Nature Med.* 2:714-6, 1996). The production of recombinant polypeptides in cultured mammalian cells is disclosed, for example, by Levinson et al., U.S. Patent No. 4,713,339; Hagen et al., U.S. Patent No. 4,784,950; Palmiter et al., U.S. Patent No. 4,579,821; and Ringold, U.S. Patent No. 4,656,134. Suitable cultured mammalian cells include the COS-1 (ATCC No. CRL 1650), COS-7 (ATCC No. CRL 1651), BHK (ATCC No. CRL 1632), BHK 570 (ATCC No. CRL 10314), 293 (ATCC No. CRL 1573; Graham et al., *J. Gen. Virol.* 36:59-72, 1977) and Chinese hamster ovary (e.g. CHO-K1; ATCC No. CCL 61) cell lines. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Manassas, VA. In general, strong transcription promoters are preferred, such as promoters from SV-40 or cytomegalovirus. See, e.g., U.S. Patent No. 4,956,288. Other suitable promoters include those from metallothionein genes (U.S. Patent Nos. 4,579,821 and 4,601,978) and the adenovirus major late promoter.

Drug selection is generally used to select for cultured mammalian cells into which foreign DNA has been inserted. Such cells are commonly referred to as "transfectants". Cells that have been cultured in the presence of the selective agent and are able to pass the gene of interest to their progeny are referred to as "stable transfectants." A preferred selectable marker is a gene encoding resistance to the antibiotic neomycin. Selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems can also be used to increase the expression level of the gene of interest, a process referred to as "amplification." Amplification is carried out by culturing transfectants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. A preferred amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (e.g. hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used. Alternative markers that introduce an altered phenotype, such as green fluorescent protein, or cell surface proteins such as CD4, CD8, Class I MHC, placental alkaline phosphatase may be used to sort transfected cells from untransfected cells by such means as FACS sorting or magnetic bead separation technology.

Other higher eukaryotic cells can also be used as hosts, including plant cells, insect cells and avian cells. The use of *Agrobacterium rhizogenes* as a vector for expressing genes in plant cells has been reviewed by Sinkar et al., *J. Biosci. (Bangalore)* 11:47-58, 1987. Transformation of insect cells and production of foreign polypeptides

therein is disclosed by Guarino et al., U.S. Patent No. 5,162,222 and WIPO publication WO 94/06463. Insect cells can be infected with recombinant baculovirus, commonly derived from *Autographa californica nuclear polyhedrosis virus* (AcNPV). See, King, L.A. and Possee, R.D., The Baculovirus Expression System: A Laboratory Guide, London, Chapman & Hall; O'Reilly, D.R. et al., Baculovirus Expression Vectors: A Laboratory Manual, New York, Oxford University Press., 1994; and, Richardson, C. D., Ed., Baculovirus Expression Protocols. Methods in Molecular Biology, Totowa, NJ, Humana Press, 1995. The second method of making recombinant baculovirus utilizes a transposon-based system described by Luckow (Luckow, V.A, et al., J Virol 67:4566-79, 1993). This system is sold in the Bac-to-Bac kit (Life Technologies, Rockville, MD). This system utilizes a transfer vector, pFastBac1™ (Life Technologies) containing a Tn7 transposon to move the DNA encoding the IL-21 polypeptide into a baculovirus genome maintained in *E. coli* as a large plasmid called a "bacmid." The pFastBac1™ transfer vector utilizes the AcNPV polyhedrin promoter to drive the expression of the gene of interest, in this case zalpha11 Ligand. However, pFastBac1™ can be modified to a considerable degree. The polyhedrin promoter can be removed and substituted with the baculovirus basic protein promoter (also known as Pcor, p6.9 or MP promoter) which is expressed earlier in the baculovirus infection, and has been shown to be advantageous for expressing secreted proteins. See, Hill-Perkins, M.S. and Possee, R.D., J. Gen. Virol. 71:971-6, 1990; Bonning, B.C. et al., J. Gen. Virol. 75:1551-6, 1994; and, Chazenbalk, G.D., and Rapoport, B., J. Biol. Chem. 270:1543-9, 1995. In such transfer vector constructs, a short or long version of the basic protein promoter can be used. Moreover, transfer vectors can be constructed which replace the native IL-21 secretory signal sequences with secretory signal sequences derived from insect proteins. For example, a secretory signal sequence from Ecdysteroid Glucosyltransferase (EGT), honey bee Melittin (Invitrogen, Carlsbad, CA), or baculovirus gp67 (PharMingen, San Diego, CA) can be used in constructs to replace the native IL-21 secretory signal sequence. In addition, transfer vectors can include an in-frame fusion with DNA encoding an epitope tag at the C- or N-terminus of the expressed IL-21 polypeptide, for example, a Glu-Glu epitope tag (Grussenmeyer, T. et al., Proc. Natl. Acad. Sci. 82:7952-4, 1985). Using techniques known in the art, a transfer vector containing IL-21 is transformed into *E. Coli*, and screened for bacmids which contain an interrupted lacZ gene indicative of recombinant baculovirus. The bacmid DNA containing the recombinant baculovirus genome is isolated, using common techniques, and used to transfect *Spodoptera frugiperda* cells, e.g. Sf9 cells. Recombinant virus that expresses IL-21 is subsequently produced. Recombinant viral stocks are made by methods commonly used the art.

The recombinant virus is used to infect host cells, typically a cell line derived from the fall armyworm, *Spodoptera frugiperda*. See, in general, Glick and Pasternak, Molecular Biotechnology: Principles and Applications of Recombinant DNA, ASM Press, Washington, D.C., 1994. Another suitable cell line is the High Five<sup>TM</sup> cell line (Invitrogen) derived from *Trichoplusia ni* (U.S. Patent #5,300,435).

Fungal cells, including yeast cells, can also be used within the present invention. Yeast species of particular interest in this regard include *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Pichia methanolica*. Methods for transforming *S. cerevisiae* cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311; Kawasaki et al., U.S. Patent No. 4,931,373; Brake, U.S. Patent No. 4,870,008; Welch et al., U.S. Patent No. 5,037,743; and Murray et al., U.S. Patent No. 4,845,075. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (e.g., leucine). A preferred vector system for use in *Saccharomyces cerevisiae* is the *POT1* vector system disclosed by Kawasaki et al. (U.S. Patent No. 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media. Suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, e.g., Kawasaki, U.S. Patent No. 4,599,311; Kingsman et al., U.S. Patent No. 4,615,974; and Bitter, U.S. Patent No. 4,977,092) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446; 5,063,154; 5,139,936 and 4,661,454. Transformation systems for other yeasts, including *Hansenula polymorpha*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Ustilago maydis*, *Pichia pastoris*, *Pichia methanolica*, *Pichia guilliermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson et al., J. Gen. Microbiol. 132:3459-65, 1986 and Cregg, U.S. Patent No. 4,882,279. *Aspergillus* cells may be utilized according to the methods of McKnight et al., U.S. Patent No. 4,935,349. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino et al., U.S. Patent No. 5,162,228. Methods for transforming *Neurospora* are disclosed by Lambowitz, U.S. Patent No. 4,486,533.

The use of *Pichia methanolica* as host for the production of recombinant proteins is disclosed in WIPO Publications WO 97/17450, WO 97/17451, WO 98/02536, and WO 98/02565. DNA molecules for use in transforming *P. methanolica* will commonly be prepared as double-stranded, circular plasmids, which are preferably linearized prior to transformation. For polypeptide production in *P. methanolica*, it is preferred that the promoter and terminator in the plasmid be that of a *P. methanolica* gene, such as a *P. methanolica* alcohol utilization gene (*AUG1* or *AUG2*). Other useful

promoters include those of the dihydroxyacetone synthase (DHAS), formate dehydrogenase (FMD), and catalase (CAT) genes. To facilitate integration of the DNA into the host chromosome, it is preferred to have the entire expression segment of the plasmid flanked at both ends by host DNA sequences. A preferred selectable marker  
5 for use in *Pichia methanolica* is a *P. methanolica* ADE2 gene, which encodes phosphoribosyl-5-aminoimidazole carboxylase (AIRC; EC 4.1.1.21), which allows *ade2* host cells to grow in the absence of adenine. For large-scale, industrial processes where it is desirable to minimize the use of methanol, it is preferred to use host cells in which both methanol utilization genes (*AUG1* and *AUG2*) are deleted. For production  
10 of secreted proteins, host cells deficient in vacuolar protease genes (*PEP4* and *PRB1*) are preferred. Electroporation is used to facilitate the introduction of a plasmid containing DNA encoding a polypeptide of interest into *P. methanolica* cells. It is preferred to transform *P. methanolica* cells by electroporation using an exponentially decaying, pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm, preferably about 3.75 kV/cm, and a time constant ( $\Omega$ ) of from 1 to 40 milliseconds,  
15 most preferably about 20 milliseconds.

Prokaryotic host cells, including strains of the bacteria *Escherichia coli*, *Bacillus* and other genera are also useful host cells within the present invention. Techniques for transforming these hosts and expressing foreign DNA sequences cloned  
20 therein are well known in the art (see, e.g., Sambrook et al., *ibid.*). When expressing a IL-21 polypeptide in bacteria such as *E. coli*, the polypeptide may be retained in the cytoplasm, typically as insoluble granules, or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed, and the granules are recovered and denatured using, for example, guanidine isothiocyanate or  
25 urea. The denatured polypeptide can then be refolded and dimerized by diluting the denaturant, such as by dialysis against a solution of urea and a combination of reduced and oxidized glutathione, followed by dialysis against a buffered saline solution. In the latter case, the polypeptide can be recovered from the periplasmic space in a soluble and functional form by disrupting the cells (by, for example, sonication or osmotic  
30 shock) to release the contents of the periplasmic space and recovering the protein, thereby obviating the need for denaturation and refolding.

Transformed or transfected host cells are cultured according to conventional procedures in a culture medium containing nutrients and other components required for the growth of the chosen host cells. A variety of suitable  
35 media, including defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals. Media may also contain such components as growth factors or serum, as

required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker carried on the expression vector or co-transfected into the host cell. *P. methanolica* cells are cultured in a medium comprising  
5 adequate sources of carbon, nitrogen and trace nutrients at a temperature of about 25°C to 35°C. Liquid cultures are provided with sufficient aeration by conventional means, such as shaking of small flasks or sparging of fermentors. A preferred culture medium for *P. methanolica* is YEPD (2% D-glucose, 2% Bacto™ Peptone (Difco Laboratories, Detroit, MI), 1% Bacto™ yeast extract (Difco Laboratories), 0.004% adenine and  
10 0.006% L-leucine).

It is preferred to purify the polypeptides of the present invention to  $\geq$  80% purity, more preferably to  $\geq$ 90% purity, even more preferably  $\geq$ 95% purity, and particularly preferred is a pharmaceutically pure state, that is greater than 99.9% pure with respect to contaminating macromolecules, particularly other proteins and nucleic  
15 acids, and free of infectious and pyrogenic agents. Preferably, a purified polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin.

Expressed recombinant IL-21 polypeptides (or chimeric IL-21 polypeptides) can be purified using fractionation and/or conventional purification methods and media. Ammonium sulfate precipitation and acid or chaotrope extraction  
20 may be used for fractionation of samples. Exemplary purification steps may include hydroxyapatite, size exclusion, FPLC and reverse-phase high performance liquid chromatography. Suitable chromatographic media include derivatized dextrans, agarose, cellulose, polyacrylamide, specialty silicas, and the like. PEI, DEAE, QAE and Q derivatives are preferred. Exemplary chromatographic media include those media  
25 derivatized with phenyl, butyl, or octyl groups, such as Phenyl-Sepharose FF (Pharmacia), Toyopearl butyl 650 (Toso Haas, Montgomeryville, PA), Octyl-Sepharose (Pharmacia) and the like; or polyacrylic resins, such as Amberchrom CG 71 (Toso Haas) and the like. Suitable solid supports include glass beads, silica-based resins, cellulosic resins, agarose beads, cross-linked agarose beads, polystyrene beads, cross-  
30 linked polyacrylamide resins and the like that are insoluble under the conditions in which they are to be used. These supports may be modified with reactive groups that allow attachment of proteins by amino groups, carboxyl groups, sulfhydryl groups, hydroxyl groups and/or carbohydrate moieties. Examples of coupling chemistries include cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide  
35 activation, sulfhydryl activation, hydrazide activation, and carboxyl and amino derivatives for carbodiimide coupling chemistries. These and other solid media are well known and widely used in the art, and are available from commercial suppliers.

Methods for binding receptor polypeptides to support media are well known in the art. Selection of a particular method is a matter of routine design and is determined in part by the properties of the chosen support. See, for example, Affinity Chromatography: Principles & Methods, Pharmacia LKB Biotechnology, Uppsala, Sweden, 1988.

5           The polypeptides of the present invention can be isolated by exploitation of their physical or biochemical properties. For example, immobilized metal ion adsorption (IMAC) chromatography can be used to purify histidine-rich proteins, including those comprising polyhistidine tags. Briefly, a gel is first charged with divalent metal ions to form a chelate (Sulkowski, Trends in Biochem. 3:1-7, 1985).  
10 Histidine-rich proteins will be adsorbed to this matrix with differing affinities, depending upon the metal ion used, and will be eluted by competitive elution, lowering the pH, or use of strong chelating agents. Other methods of purification include purification of glycosylated proteins by lectin affinity chromatography and ion exchange chromatography (Methods in Enzymol., Vol. 182, "Guide to Protein  
15 Purification", M. Deutscher, (ed.), Acad. Press, San Diego, 1990, pp.529-39) and use of the soluble  $\alpha$ 11 receptor. Within additional embodiments of the invention, a fusion of the polypeptide of interest and an affinity tag (e.g., maltose-binding protein, an immunoglobulin domain) may be constructed to facilitate purification.

IL-21 polypeptides or fragments thereof may also be prepared through  
20 chemical synthesis. IL-21 polypeptides may be monomers or multimers; glycosylated or non-glycosylated; pegylated or non-pegylated; and may or may not include an initial methionine amino acid residue. For example, the polypeptides can be prepared by solid phase peptide synthesis, for example as described by Merrifield, J. Am. Chem. Soc. 85:2149, 1963.

25           The activity of molecules of the present invention can be measured using a variety of assays that measure proliferation of and/or binding to cells expressing the IL-21 receptor. Of particular interest are changes in IL-21-dependent cells. Suitable cell lines to be engineered to be IL-21-dependent include the IL-3-dependent BaF3 cell line (Palacios and Steinmetz, Cell 41: 727-734, 1985; Mathey-Prevot et al., Mol. Cell. Biol.  
30 6: 4133-4135, 1986), FDC-P1 (Hapel et al., Blood 64: 786-790, 1984), and MO7e (Kiss et al., Leukemia 7: 235-240, 1993). Growth factor-dependent cell lines can be established according to published methods (e.g. Greenberger et al., Leukemia Res. 8: 363-375, 1984; Dexter et al., in Baum et al. Eds., Experimental Hematology Today, 8th  
Ann. Mtg. Int. Soc. Exp. Hematol. 1979, 145-156, 1980).

35           Proteins of the present invention are useful for induction or inhibition of specialized cell functions of cells of the hematopoietic lineages, including, but not limited to, T cells, B cells, NK cells, dendritic cells, monocytes, and macrophages, as

well as epithelial cells. IL-21 antagonists are used to block IL-21 binding and signal transduction *in vitro* and *in vivo*. These anti-IL-21 binding polypeptides would be useful for inhibiting IL-21 activity or protein-binding. Assays measuring cell proliferation or differentiation are well known in the art. For example, assays measuring proliferation include such assays as chemosensitivity to neutral red dye (Cavanaugh et al., Investigational New Drugs 8:347-354, 1990, incorporated herein by reference), incorporation of radiolabelled nucleotides (Cook et al., Analytical Biochem. 179:1-7, 1989, incorporated herein by reference), incorporation of 5-bromo-2'-deoxyuridine (BrdU) in the DNA of proliferating cells (Porstmann et al., J. Immunol. Methods 82:169-179, 1985, incorporated herein by reference), and use of tetrazolium salts (Mosmann, J. Immunol. Methods 65:55-63, 1983; Alley et al., Cancer Res. 48:589-601, 1988; Marshall et al., Growth Reg. 5:69-84, 1995; and Scudiero et al., Cancer Res. 48:4827-4833, 1988; all incorporated herein by reference). Assays measuring differentiation include, for example, measuring cell-surface markers associated with stage-specific expression of a tissue, enzymatic activity, functional activity or morphological changes (Watt, FASEB, 5:281-284, 1991; Francis, Differentiation 57:63-75, 1994; Raes, Adv. Anim. Cell Biol. Technol. Bioprocesses, 161-171, 1989; all incorporated herein by reference).

The molecules of the present invention can be assayed *in vivo* using viral delivery systems. Exemplary viruses for this purpose include adenovirus, herpesvirus, retroviruses, vaccinia virus, and adeno-associated virus (AAV). Adenovirus, a double-stranded DNA virus, is currently the best studied gene transfer vector for delivery of heterologous nucleic acid (for review, see T.C. Becker et al., Meth. Cell Biol. 43:161-89, 1994; and J.T. Douglas and D.T. Curiel, Science & Medicine 4:44-53, 1997).

In view of the tissue distribution observed for IL-21 receptor agonists (including the natural IL-21/ substrate/ cofactor/ etc.) and/or antagonists have enormous potential in both *in vitro* and *in vivo* applications. Antagonists are useful as research reagents for characterizing sites of ligand-receptor interaction. Antagonists are useful to inhibit expansion, proliferation, activation, and/or differentiation of cells involved in regulating hematopoiesis.

An assay system that uses a ligand-binding receptor (or an antibody, one member of a complement/ anti-complement pair) or a binding fragment thereof, and a commercially available biosensor instrument (BIAcore, Pharmacia Biosensor, Piscataway, NJ) may be advantageously employed. Such receptor, antibody, member of a complement/anti-complement pair or fragment is immobilized onto the surface of a receptor chip. Use of this instrument is disclosed by Karlsson, J. Immunol. Methods 145:229-40, 1991 and Cunningham and Wells, J. Mol. Biol. 234:554-63, 1993. A

receptor, antibody, member or fragment is covalently attached, using amine or sulfhydryl chemistry, to dextran fibers that are attached to gold film within the flow cell. A test sample is passed through the cell. If a ligand, epitope, or opposite member of the complement/anti-complement pair is present in the sample, it will bind to the immobilized receptor, antibody or member, respectively, causing a change in the refractive index of the medium, which is detected as a change in surface plasmon resonance of the gold film. This system allows the determination of on- and off-rates, from which binding affinity can be calculated, and assessment of stoichiometry of binding. Alternatively, ligand/receptor binding can be analyzed using SELDI(TM) technology (Ciphergen, Inc., Palo Alto, CA).

Ligand-binding receptor polypeptides can also be used within other assay systems known in the art. Such systems include Scatchard analysis for determination of binding affinity (see Scatchard, Ann. NY Acad. Sci. 51: 660-72, 1949) and calorimetric assays (Cunningham et al., Science 253:545-48, 1991; Cunningham et al., Science 245:821-25, 1991).

IL-21 antagonist polypeptides can also be used to prepare antibodies that bind to IL-21 epitopes, peptides or polypeptides. The IL-21 polypeptide or a fragment thereof serves as an antigen (immunogen) to inoculate an animal and elicit an immune response. One of skill in the art would recognize that antigenic, epitope-bearing polypeptides contain a sequence of at least 6, preferably at least 9, and more preferably at least 15 to about 30 contiguous amino acid residues of a IL-21 polypeptide (e.g., SEQ ID NOS:4 or 6). Polypeptides comprising a larger portion of IL-21 antagonist polypeptide, i.e., from 30 to 100 residues up to the entire length of the amino acid sequence are included. Antigens or immunogenic epitopes can also include attached tags, adjuvants and carriers, as described herein. Suitable antigens include the full length and the mature IL-21 antagonist polypeptide, mutant helix D, as described herein.

IL-21 antagonist fusion proteins can be used for enhancing *in vivo* killing of target tissues (for example, blood and bone marrow cancers), if the IL-21 polypeptide or anti-IL-21 antibody targets the hyperproliferative blood or bone marrow cell (See, generally, Hornick et al., Blood 89:4437-47, 1997). The described fusion proteins enable targeting of a cytokine to a desired site of action, thereby providing an elevated local concentration of cytokine. Suitable IL-21 polypeptides or anti-IL-21 antibodies target an undesirable cell or tissue (i.e., a tumor or a leukemia), and the fused cytokine mediated improved target cell lysis by effector cells. Suitable cytokines for this purpose include interleukin 2 and granulocyte-macrophage colony-stimulating factor (GM-CSF), for instance.



IL-21 was isolated from tissue known to have important immunological function and which contain cells that play a role in the immune system. IL-21 is expressed in CD3+ selected, activated peripheral blood cells, and it has been shown that IL-21 expression increases after T cell activation. Moreover, results of experiments have previously demonstrated that IL-21 polypeptides have an effect on the growth/expansion and/or differentiated state of NK cells or NK progenitors. Additional evidence demonstrated that IL-21 affected proliferation and/or differentiation of T cells and B cells *in vivo*. Factors that both stimulate proliferation of hematopoietic progenitors and activate mature cells are generally known. Thus, IL-21 antagonist polypeptides will be useful for inhibiting the growth and differentiation of these IL-21-responsive cell types. This is particularly useful when proliferation of a specific responsive cell type is associated with a hyper-proliferative disease, such as cancer or autoimmune disease.

Assays measuring differentiation include, for example, measuring cell markers associated with stage-specific expression of a tissue, enzymatic activity, functional activity or morphological changes (Watt, FASEB, 5:281-284, 1991; Francis, Differentiation 57:63-75, 1994; Raes, Adv. Anim. Cell Biol. Technol. Bioprocesses, 161-171, 1989; all incorporated herein by reference). Alternatively, IL-21 polypeptide itself can serve as an additional cell-surface or secreted marker associated with stage-specific expression of a tissue. As such, direct measurement of IL-21 polypeptide, or its loss of expression in a tissue as it differentiates, can serve as a marker for differentiation of tissues.

The activity and effect of IL-21 antagonists on tumor progression and metastasis can be measured *in vivo*. Several syngeneic mouse models have been developed to study the influence of polypeptides, compounds or other treatments on tumor progression. In these models, tumor cells passaged in culture are implanted into mice of the same strain as the tumor donor. The cells will develop into tumors having similar characteristics in the recipient mice, and metastasis will also occur in some of the models. Appropriate tumor models for our studies include the Lewis lung carcinoma (ATCC No. CRL-1642) and B16 melanoma (ATCC No. CRL-6323), amongst others. These are both commonly used tumor lines, syngeneic to the C57BL6/J mouse, that are readily cultured and manipulated *in vitro*. Tumors resulting from implantation of either of these cell lines are capable of metastasis to the lung in C57BL6/J mice. The Lewis lung carcinoma model has recently been used in mice to identify an inhibitor of angiogenesis (O'Reilly MS, et al. Cell 79: 315-328, 1994). C57BL6/J mice are treated with an experimental agent either through daily injection of recombinant protein, agonist or antagonist or a one time injection of recombinant

adenovirus. Three days following this treatment,  $10^5$  to  $10^6$  cells are implanted under the dorsal skin. Alternatively, the cells themselves may be infected with recombinant adenovirus, such as one expressing IL-21, before implantation so that the protein is synthesized at the tumor site or intracellularly, rather than systemically. The mice normally develop visible tumors within 5 days. The tumors are allowed to grow for a period of up to 3 weeks, during which time they may reach a size of 1500 - 1800 mm<sup>3</sup> in the control treated group. Tumor size and body weight are carefully monitored throughout the experiment. At the time of sacrifice, the tumor is removed and weighed along with the lungs and the liver. The lung weight has been shown to correlate well with metastatic tumor burden. As an additional measure, lung surface metastases are counted. The resected tumor, lungs and liver are prepared for histopathological examination, immunohistochemistry, and *in situ* hybridization, using methods known in the art and described herein. The influence of the expressed polypeptide in question, e.g., IL-21, on the ability of the tumor to recruit vasculature and undergo metastasis can thus be assessed. In addition, aside from using adenovirus, the implanted cells can be transiently transfected with IL-21. Use of stable IL-21 transfectants as well as use of inducible promoters to activate IL-21 expression *in vivo* are known in the art and can be used in this system to assess IL-21 induction of metastasis. Moreover, purified IL-21 or IL-21 conditioned media can be directly injected in to this mouse model, and hence be used in this system. For general reference see, O'Reilly MS, et al. Cell 79:315-328, 1994; and Rusciano D, et al. Murine Models of Liver Metastasis. Invasion Metastasis 14:349-361, 1995.

IL-21 antagonists could be administered in combination with other agents already in use including both conventional chemotherapeutic agents as well as immune modulators such as interferon alpha. Alpha/beta interferons have been shown to be effective in treating some leukemias and animal disease models, and the growth inhibitory effects of interferon-alpha and IL-21 are additive for at least one B-cell tumor-derived cell line.

In another aspect, the present invention provides a method of reducing proliferation of a neoplastic B or T cells comprising administering to a mammal with a B or T cell neoplasm an amount of a composition of IL-21 antagonist sufficient to reducing proliferation of the neoplastic B or T cells. In other embodiments, the composition can comprise at least one other cytokine selected from the group consisting of IL-2, IL-15, IL-4, GM-CSF, Flt3 ligand or stem cell factor. Furthermore, the IL-21 antagonist can be a ligand/toxin fusion protein.

The tissue distribution of a receptor for a given cytokine offers a strong indication of the potential sites of action of that cytokine. Northern analysis of IL-21

receptor revealed transcripts in human spleen, thymus, lymph node, bone marrow, and peripheral blood leukocytes. Specific cell types were identified as expressing  $\alpha 11$  receptors, and strong signals were seen in a mixed lymphocyte reaction (MLR) and in the Burkitt's lymphoma Raji. The two monocytic cell lines, THP-1 (Tsuchiya et al., Int. J. Cancer 26:171-176, 1980) and U937 (Sundstrom et al., Int. J. Cancer 17:565-577, 1976), were negative.

IL-21 receptor is expressed at relatively high levels in the MLR, in which peripheral blood mononuclear cells (PBMNC) from two individuals are mixed, resulting in mutual activation. Detection of high levels of transcript in the MLR but not in resting T or B cell populations suggests that IL-21 receptor expression may be induced in one or more cell types during activation. Activation of isolated populations of T and B cells can be artificially achieved by stimulating cells with PMA and ionomycin. When sorted cells were subjected to these activation conditions, levels of IL-21 receptor transcript increased in both cell types, supporting a role for this receptor and IL-21 in immune responses, especially in autocrine and paracrine T and B cell expansions during activation. IL-21 may also play a role in the expansion of more primitive progenitors involved in lymphopoiesis.

IL-21 receptor was found to be present at low levels in resting T and B cells, and was upregulated during activation in both cell types. Interestingly, the B cells also down-regulate the message more quickly than do T cells, suggesting that amplitude of signal and timing of quenching of signal are important for the appropriate regulation of B cell responses.

In addition, a large proportion of intestinal lamina propria cells show positive hybridization signals with IL-21 receptor. This tissue consists of a mixed population of lymphoid cells, including activated CD4<sup>+</sup> T cells and activated B cells. Immune dysfunction, in particular chronic activation of the mucosal immune response, plays an important role in the etiology of Crohn's disease; abnormal response to and/or production of proinflammatory cytokines is also a suspected factor (Braegger et al., Annals Allergy 72:135-141, 1994; Sartor RB Am. J. Gastroenterol. 92:5S-11S, 1997).

IL-21 in concert with IL-15 expands NK cells from bone marrow progenitors and augments NK cell effector function. IL-21 also co-stimulates mature B cells stimulated with anti-CD40 antibodies, but inhibits B cell proliferation to signals through IgM. IL-21 enhances T cell proliferation in concert with a signal through the T cell receptor, and overexpression in transgenic mice leads to lymphopenia and an expansion of monocytes and granulocytes. These pleiotropic effects of IL-21 suggest that it can provide therapeutic utility for a wide range of diseases arising from defects in the immune system, including (but not limited to) systemic lupus erythematosus (SLE), rheumatoid

arthritis (RA), multiple sclerosis (MS), myasthenia gravis, and diabetes. It is important to note that these diseases are the result of a complex network of immune dysfunction (SLE, for example, is the manifestation of defects in both T and B cells), and that immune cells are dependent upon interaction with one another to elicit a potent immune response. Therefore, IL-21 antagonist that can be used to manipulate more than one type of immune cell is an attractive therapeutic candidate for intervention at multiple stages of disease.

For pharmaceutical use, the proteins of the present invention are formulated for parenteral, particularly intravenous or subcutaneous, delivery according to conventional methods. The bioactive polypeptide or antibody conjugates described herein can be delivered intravenously, intraarterially or intraductally, or may be introduced locally at the intended site of action. Intravenous administration will be by bolus injection or infusion over a typical period of one to several hours. In general, pharmaceutical formulations will include a IL-21 protein in combination with a pharmaceutically acceptable vehicle, such as saline, buffered saline, 5% dextrose in water or the like. Formulations may further include one or more excipients, preservatives, solubilizers, buffering agents, albumin to prevent protein loss on vial surfaces, etc. Methods of formulation are well known in the art and are disclosed, for example, in Remington: The Science and Practice of Pharmacy, Gennaro, ed., Mack Publishing Co., Easton, PA, 19th ed., 1995. Therapeutic doses will generally be in the range of 0.1 to 100  $\mu\text{g/kg}$  of patient weight per day, preferably 0.5-20  $\mu\text{g/kg}$  per day, with the exact dose determined by the clinician according to accepted standards, taking into account the nature and severity of the condition to be treated, patient traits, etc. Determination of dose is within the level of ordinary skill in the art. The proteins may be administered for acute treatment, over one week or less, often over a period of one to three days or may be used in chronic treatment, over several months or years. In general, a therapeutically effective amount of IL-21 is an amount sufficient to produce a clinically significant change in hematopoietic or immune function.

The invention is further illustrated by the following non-limiting examples.

EXAMPLESExample 1Construction of MPL-IL-21 Receptor Polypeptide Chimera: MPL Extracellular and  
5 TM Domain Fused to the IL-21 Receptor Intracellular Signaling Domain

The extracellular and transmembrane domains of the murine MPL receptor were isolated from a plasmid containing the murine MPL receptor (PHZ1/MPL plasmid) using PCR with primers ZC17,212 (SEQ ID NO:11) and ZC19,914 (SEQ ID NO:12). The reaction conditions were as follows: 95°C for 1 min.; 35 cycles at 95°C  
10 for 1 min., 45°C for 1 min., 72°C for 2 min.; followed by 72°C at 10 min.; then a 10°C soak. The PCR product was run on a 1% low melting point agarose (Boehringer Mannheim, Indianapolis, IN) and the approximately 1.5 kb MPL receptor fragment isolated using Qiaquick™ gel extraction kit (Qiagen) as per manufacturer's instructions.

15 The intracellular domains of human IL-21 receptor were isolated from a plasmid containing IL-21 receptor cDNA using PCR with primers ZC19,913 (SEQ ID NO:13) and ZC20,097 (SEQ ID NO:14). The polynucleotide sequence corresponding to the IL-21 receptor coding sequence is shown in SEQ ID NO:15, and the corresponding amino acid sequence shown in SEQ ID NO:16. The reaction conditions  
20 were as per above. The PCR product was run on a 1% low melting point agarose (Boehringer Mannheim) and the approximately 900 bp IL-21 receptor fragment isolated using Qiaquick gel extraction kit as per manufacturer's instructions.

Each of the isolated fragments described above were mixed at a 1:1 volumetric ratio and used in a PCR reaction using ZC17,212 (SEQ ID NO:11) and  
25 ZC20,097 (SEQ ID NO:14) to create the MPL-IL-21 receptor chimera. The reaction conditions were as follows: 95°C for 1 min.; 35 cycles at 95°C for 1 min., 55°C for 1 min., 72°C for 2 min.; followed by 72°C at 10 min.; then a 10°C soak. The entire PCR product was run on a 1% low melting point agarose (Boehringer Mannheim) and the approximately 2.4 kb MPL-IL-21 receptor chimera fragment isolated using Qiaquick  
30 gel extraction kit (Qiagen) as per manufacturer's instructions. The MPL-IL-21 receptor chimera fragment was digested with EcoRI (BRL) and XbaI (Boehringer Mannheim) as per manufacturer's instructions. The entire digest was run on a 1% low melting point agarose (Boehringer Mannheim) and the cleaved MPL-IL-21 receptor chimera isolated using Qiaquick™ gel extraction kit (Qiagen) as per manufacturer's instructions. The  
35 resultant cleaved MPL-IL-21 receptor chimera was inserted into an expression vector as described below.

Recipient expression vector pZP-5N was digested with EcoRI (BRL) and HindIII (BRL) as per manufacturer's instructions, and gel purified as described above. This vector fragment was combined with the EcoRI and XbaI cleaved MPL-IL-21 receptor chimera isolated above and a XbaI/HindIII linker fragment in a ligation reaction. The ligation was run using T4 Ligase (BRL), at 15°C overnight. A sample of the ligation was electroporated in to DH10B ElectroMAX™ electrocompetent *E. coli* cells (25µF, 200Ω, 2.3V). Transformants were plated on LB+Ampicillin plates and single colonies screened by PCR to check for the MPL-IL-21 receptor chimera using ZC17,212 (SEQ ID NO:11) and ZC20,097 (SEQ ID NO:14) using the PCR conditions as described above.

### Example 2

#### MPL-IL-21 Receptor Chimera-based Proliferation in BAF3 Assay Using Alamar Blue

##### A. Construction of BaF3 Cells Expressing MPL-IL-21 Receptor Chimera

BaF3, an interleukin-3 (IL-3) dependent pre-lymphoid cell line derived from murine bone marrow (Palacios and Steinmetz, Cell 41: 727-734, 1985; Mathey-Prevot et al., Mol. Cell. Biol. 6: 4133-4135, 1986), was maintained in complete media (RPMI medium (JRH Bioscience Inc., Lenexa, KS) supplemented with 10% heat-inactivated fetal calf serum, 2ng/ml murine IL-3 (mIL-3) (R & D, Minneapolis, MN), 2mM L-glutaMax-1™ (Gibco BRL), 1 mM Sodium Pyruvate (Gibco BRL), and PSN antibiotics (GIBCO BRL)). Prior to electroporation, pZP-5N/MPL-IL-21 receptor plasmid DNA (Example 1) was prepared and purified using a Qiagen Maxi Prep kit (Qiagen) as per manufacturer's instructions. BaF3 cells for electroporation were washed once in RPMI media and then resuspended in RPMI media at a cell density of 10<sup>7</sup> cells/ml. One ml of resuspended BaF3 cells was mixed with 30 µg of the pZP-5N/MPL-IL-21 receptor plasmid DNA and transferred to separate disposable electroporation chambers (GIBCO BRL). Following a 15 minute incubation at room temperature the cells were given two serial shocks (800 IFad/300 V.; 1180 IFad/300 V.) delivered by an electroporation apparatus (CELL-PORATOR™; GIBCO BRL). After a 5 minute recovery time, the electroporated cells were transferred to 50 ml of complete media and placed in an incubator for 15-24 hours (37°C, 5% CO<sub>2</sub>). The cells were then spun down and resuspended in 50 ml of complete media containing Geneticin™ (Gibco) selection (500 µg/ml G418) in a T-162 flask to isolate the G418-resistant pool. Pools of the transfected BaF3 cells, hereinafter called BaF3/MPL-IL-21 receptor cells, were assayed for signaling capability as described below.

Example 3Construction of Expression Vector Expressing Full-length IL-21Receptor

The entire IL-21 receptor was isolated from a plasmid containing  
5 zalpha11 receptor cDNA (SEQ ID NO:15) using PCR with primers ZC19,905 (SEQ ID  
NO:19) and ZC19,906 (SEQ ID NO:20). The reaction conditions were as follows:  
95°C for 1 min; 35 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 2 min; followed  
by 72°C at 10 min; then a 10°C soak. The PCR product was run on a 1% low melting  
point agarose (Boehringer Mannheim) gel and the approximately 1.5 kb zalpha11  
10 cDNA isolated using Qiaquick™ gel extraction kit (Qiagen) as per manufacturer's  
instructions.

The purified IL-21 receptor cDNA was digested with BamHI  
(Boehringer Mannheim) and EcoRI (BRL) as per manufacturer's instructions. The  
entire digest was run on a 1% low melting point agarose (Boehringer Mannheim) gel  
15 and the cleaved IL-21 receptor fragment was purified the using Qiaquick™ gel  
extraction kit as per manufacturer's instructions. The resultant cleaved IL-21 receptor  
fragment was inserted into an expression vector as described below.

Recipient expression vector pZP-5N was digested with BamHI  
(Boehringer Mannheim) and EcoRI (BRL) as per manufacturer's instructions, and gel  
20 purified as described above. This vector fragment was combined with the BamHI and  
EcoRI cleaved IL-21 receptor fragment isolated above in a ligation reaction using T4  
Ligase (BRL). The ligation was incubated at 15°C overnight. A sample of the ligation  
was electroporated in to DH10B electroMAX™ electrocompetent *E. coli* cells (25μF,  
200Ω, 2.3V). Transformants were plated on LB+Ampicillin plates and single colonies  
25 screened by PCR to check for the IL-21 receptor sequence using ZC19,905 (SEQ ID  
NO:19) and ZC19,906 (SEQ ID NO:20) using the PCR conditions as described above.

Example 4IL-21 Activates Human IL-21 Receptor in Luciferase Assay30 A. Construction of BaF3/KZ134/IL-21 receptor cell line

The KZ134 plasmid was constructed with complementary  
oligonucleotides ZC12,749 (SEQ ID NO:17) and ZC12,748 (SEQ ID NO:18) that  
contain STAT transcription factor binding elements from 4 genes. A modified c-fos Sis  
inducible element (m67SIE, or hSIE) (Sadowski, H. et al., *Science* 261:1739-1744,  
35 1993), the p21 SIE1 from the p21 WAF1 gene (Chin, Y. et al., *Science* 272:719-722,  
1996), the mammary gland response element of the β-casein gene (Schmitt-Ney, M. et

al., Mol. Cell. Biol. 11:3745-3755, 1991), and a STAT inducible element of the Fcg RI gene, (Seidel, H. et al., Proc. Natl. Acad. Sci. 92:3041-3045, 1995). These oligonucleotides contain Asp718-XhoI compatible ends and were ligated, using standard methods, into a recipient firefly luciferase reporter vector with a c-fos promoter (Poulsen, L.K. et al., J. Biol. Chem. 273:6229-6232, 1998) digested with the same enzymes and containing a neomycin selectable marker. The KZ134 plasmid was used to stably transfect BaF3 cells, using standard transfection and selection methods, to make the BaF3/KZ134 cell line.

A stable BaF3/KZ134 indicator cell line, expressing the full-length IL-21 receptor was constructed as per Example 2, using about 30µg of the IL-21 receptor expression vector, described in Example 3. Clones were diluted, plated and selected using standard techniques. Clones were screened by luciferase assay using the human IL-21 conditioned media as an inducer. Clones with the highest luciferase response (via STAT luciferase) and the lowest background were selected. A stable transfectant cell line was selected. The cell line was called BaF3/KZ134/IL-21 receptor.

#### B. Human and mouse IL-21 activates human IL-21 receptor in BaF3/KZ134/IL-21 receptor luciferase assay

BaF3/KZ134/ IL-21 receptor cells were spun down and washed in mouse IL-3 free media. The cells were spun and washed 3 times to ensure removal of mouse IL-3. Cells were then counted in a hemacytometer. Cells were plated in a 96-well format at about 30,000 cells per well in a volume of 100 µl per well using the mouse IL-3 free media. The same procedure was used for untransfected BaF3/KZ134 cells for use as a control in the subsequent assay.

STAT activation of the BaF3/KZ134/IL-21 receptor cells was assessed using conditioned media from (1) BHK570 cells transfected with the human IL-21 receptor or (2) BHK570 cells transfected with the mouse IL-21 receptor, or (4) mIL-3 free media to measure media-only control response. Conditioned media was diluted with RPMI mIL-3 free media to 50%, 25%, 12.5%, 6.25%, 3.125%, 1.5%, 0.75% and 0.375% concentrations. 100 µl of the diluted conditioned media was added to the BaF3/KZ134/IL-21 receptor cells. The assay using the conditioned media was done in parallel on untransfected BaF3/KZ134 cells as a control. The total assay volume was 200 µl. The assay plates were incubated at 37°C, 5% CO<sub>2</sub> for 24 hours at which time the cells were pelleted by centrifugation at 2000 rpm for 10 min., and the media was aspirated and 25 µl of lysis buffer (Promega) was added. After 10 minutes at room temperature, the plates were measured for activation of the STAT reporter construct by



reading them on a luminometer (Labsystems Luminoskan, model RS) which added 40  $\mu$ l of luciferase assay substrate (Promega) at a five second integration.

Results confirmed the STAT reporter response of the BaF3/KZ134/ IL-21 receptor cells to the human IL-21. The response, as measured, was approximately 50 fold over media-only control at the 50% concentration. STAT activation in response to human IL-21 was absent in the untransfected BaF3/KZ134 control cells, showing that the response is mediated through the IL-21 receptor.

Results also confirmed the STAT reporter response of the BaF3/KZ134/IL-21 receptor cells to the mouse IL-21. The response, as measured, was approximately 40 fold over media-only control at the 50% concentration. Moreover, STAT activation in response to mouse IL-21 was evident (about 5-fold) on the untransfected BaF/KZ134 control cells; suggesting that the murine BaF3 cells may have endogenous mouse receptor.

#### Example 5

#### Expression Vector Construction, Expression and Purification Of Untagged Human And Murine IL-21 From Baculovirus.

##### A. Construct for Expressing human IL-21 in Baculovirus

An expression vector, p $\alpha$ 11L, was prepared to express Human IL-21 polypeptides in insect cells. A 517 bp fragment containing sequence for Human IL-21 and encoded BamHI and XhoI restriction sites on the 5' and 3' ends respectively, was generated by PCR amplification from a plasmid containing human IL-21 cDNA using primers ZC23,444 (SEQ ID NO:21) and ZC23,445 (SEQ ID NO:22). The PCR reaction conditions were as follows: 1 cycle of 94°C for 4 minutes, followed by 25 cycles of 94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 2 minutes; 1 cycle at 72°C for 10 min; followed by a 4°C soak. The fragment was visualized by gel electrophoresis (1% SeaPlaque/1% NuSieve). The band was excised, diluted to 0.5% agarose with 2 mM MgCl<sub>2</sub>, melted at 65°C, digested with BamHI and XhoI (Boehringer Mannheim), and ligated into an BamHI/XhoI digested baculovirus expression vector, pZBV3L. The pZBV3L vector is a modification of the pFastBac1™ (Life Technologies) expression vector, where the polyhedron promoter has been removed and replaced with the late activating Basic Protein Promoter. About 14 nanograms of the restriction digested IL-21 insert and about 40 ng of the corresponding vector were ligated overnight at 16°C.

The ligation mix was diluted 3 fold in TE (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA) and about 4 fmol of the diluted ligation mix was transformed into DH5 $\alpha$

Library Efficiency competent cells (Life Technologies) according to manufacturer's direction by heat shock for 45 seconds in a 42°C waterbath. The transformed DNA and cells were diluted in 450 µl of SOC media (2% Bacto™ Tryptone, 0.5% Bacto Yeast Extract, 10 ml 1M NaCl, 1.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub> and 20 mM glucose) and plated onto LB plates containing 100 µg/ml ampicillin. Clones were analyzed by restriction digests and 1 µl of the positive clone was transformed into 20 µl DH10Bac Max Efficiency competent cells (GIBCO-BRL, Gaithersburg, MD) according to manufacturer's instruction, by heat shock as described above. The transformed cells were then diluted in 980 µl SOC media (2% Bacto™ Tryptone, 0.5% Bacto™ Yeast Extract, 10 ml 1M NaCl, 1.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub> and 20 mM glucose) out grown in shaking incubator at 37°C for four hours and plated onto Luria Agar plates containing 50 µg/ml kanamycin, 7 µg/ml gentamicin (Life Technologies), 10 µg/ml tetracycline, IPTG (Pharmacia Biotech) and Blue-Gal (Life Technologies). The plated cells were incubated for 48 hours at 37°C. A color selection was used to identify those cells having Human IL-21 encoding donor insert that had incorporated into the plasmid (referred to as a "bacmid"). Those colonies, which were white in color, were picked for analysis. Human IL-21 Bacmid DNA was isolated from positive colonies using the QiaVac Miniprep8 system (Qiagen) according the manufacturer's directions. Clones were screened for the correct insert by amplifying DNA using primers to the transposable element in the bacmid via PCR using primers ZC447 (SEQ ID NO:23) and ZC976 (SEQ ID NO:24). The PCR reaction conditions were as follows: 35 cycles of 94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 5 minutes; 1 cycle at 72°C for 10 min.; followed by 4C soak. The PCR product was run on a 1% agarose gel to check the insert size. Those clones having the correct insert were used to transfect *Spodoptera frugiperda* (Sf9) cells.

B. Expression and generation of material for purification of human IL-21 from baculovirus

Sf9 cells were seeded at  $5 \times 10^6$  cells per 35 mm plate and allowed to attach for 1 hour at 27°C. Five microliters of human IL-21 bacmid DNA (above) was diluted with 100 µl Sf-900 II SFM (Life Technologies). Six µl of CelIFECTIN Reagent (Life Technologies) was diluted with 100 µl Sf-900 II SFM. The bacmid DNA and lipid solutions were gently mixed and incubated 30-45 minutes at room temperature. The media from one plate of cells were aspirated, the cells were washed 1X with 2 ml fresh Sf-900 II SFM media. Eight hundred microliters of Sf-900 II SFM was added to the lipid-DNA mixture. The wash media was aspirated and the DNA-lipid mix added

to the cells. The cells were incubated at 27°C for 4-5 hours. The DNA-lipid mix was aspirated and 2 ml of Sf-900 II media was added to each plate. The plates were incubated at 27°C, 90% humidity, for 96 hours after which the virus was harvested.

For Primary Amplification Sf9 cells were grown in 50 ml Sf-900 II SFM in a 125 ml shake flask to an approximate density of  $0.41-0.52 \times 10^5$  cells/ml. They were then infected with 150  $\mu$ l of the virus stock from above and incubated at 27°C for 3 days after which time the virus was harvested according to standard methods known in the art. A 500  $\mu$ l sample submitted for activity in a BaF3 assay to show that it was biologically active.

For Secondary Amplification Sf9 cells were grown in 1L of Sf-900 II SFM in a 2800 ml shake flask to an approximate density of  $0.5 \times 10^5$  cells/ml. It was infected with 500ul of the Primary viral stock from above and incubated at 27°C for 4 days after which time the virus was harvested according to standard methods known in the art. Virus was titered and grown up large scale for purification of the baculovirus-produced human IL-21 (huzalpha11L-Bv).

#### C. Large-scaled purification of baculovirus expressed human/murine IL-21

Unless otherwise stated, all operations were carried out at 4°C. The following procedure was used for purifying human IL-21 (huzalpha11L-Bv) from BV conditioned media. Conditioned media (CM) was sterile filtered through 0.45 and 0.22 micron filters, then buffered with 0.01 M MES (Fluka BioChemika, Switzerland) and the pH adjusted to 6.0. The CM was then loaded onto a POROS 50 HS column and run, fractions collected and analyzed.

The above peak fractions were pooled, concentrated run on a high resolution size exclusion column, and analyzed.

The fractions of interest from the size exclusion column were pooled and concentrated with 5 kD MWCO Millipore centrifugal spin concentrators to a minimal volume. The final product was then analyzed by SDS-PAGE Coomassie (Sigma, St. Louis, MO), Western immunological blotting, N-terminal sequencing, Amino Acid Analysis, and CB (Pierce, Rockford, Illinois) for protein purity and concentration as described in Example 29A. Bulk protein was stored at -80°C.

#### D. Small scale (<2 mg) Purification of Baculovirus-expressed human/murine IL-21

Unless other wise stated, all operations were carried out at 4°C. The following procedure was used for purifying < 2 mg of human or murine IL-21 from BV conditioned media. The CM was filtered, buffered and pH adjusted as in Example 30C.

The CM was then loaded, eluted and the POROS 50 HS chromatography was analyzed as in Example 30C.

Fractions were pooled then concentrated via diafiltration in a stirred cell concentrator on a YM10 membrane (10 kD MWCO) (Millipore/Amicon, Bedford, MA) to a nominal volume (20-30 ml). The pH was adjusted to 7.0 then the sample was loaded onto either a 0.8 ml Poros AL column that had about 3 mg of  $\alpha$ 11CFLAG soluble receptor or one with about 10 mg of IL-21 -Fc4 fusion soluble receptor immobilized on the resin at 1 ml/min on a BioCad SPRINT. The column was then washed with at least 20 CV of 0.3 M NaCl/PBS(Gibco BRL) /0.01 M MES at 10 ml/min. The column was then rapid eluted with a 600  $\mu$ l injection of 0.1 M glycine (Aminoacetic Acid; Glycocol, Spectrum, Gardena, CA) pH 2.5 at a flow rate of 10 ml/min with PBS on a BioCAD SPRINT. The 1 ml fractions were collected for 6 seconds each and immediately pH neutralized with 55  $\mu$ l of 2 M TRIS (Tris (Hydroxymethyl) Aminomethane, EM Science, Gibbstown, NJ) pH 8.8. The absorbance at 280 and 215 nM were monitored over the entire chromatography. Fractions were analyzed as above.

Peak fractions were pooled then concentrated via diafiltration in a stirred cell concentrator on a YM10 membrane (10 kD MWCO) (Millipore/Amicon, Bedford, MA) to 1-2 ml. The sample was then loaded on an appropriate Sephacryl S-200 (Pharmacia, Uppsala, Sweden) high resolution size exclusion column equilibrated in PBS (Gibco BRL) at an optimal flow rate; fractions were collected over the entire chromatography and absorbance at 280 and 215 nM were monitored. Fractions were analyzed as above.

The fractions of interest were pooled and concentrated with 5 Kd MWCO Millipore centrifugal spin concentrators to a nominal volume. The final product was then analyzed by SDS-PAGE Coomassie (Sigma, St. Louis, MO), Western immunological blotting, N-terminal sequencing, Amino Acid Analysis, and BCA (Pierce, Rockford, Illinois) for protein purity and concentration. Bulk protein stored as described above.

### Example 6

#### IL-21-dependent Proliferation of B-cell cells Stimulated Anti-CD40 or Anti-IgM

##### A. Purification of Human B cells

A vial containing  $1 \times 10^8$  frozen, apheresed human peripheral blood mononuclear cells (PBMCs) was quickly thawed in a 37°C water bath and resuspended in 25 ml B cell medium (RPMI Medium 1640 (JRH Biosciences, Lenexa, KS), 10%

Heat inactivated fetal bovine serum, 5% L-glutamine, 5% Pen/Strep) (Gibco BRL)) in a 50 ml tube (Falcon VWR, Seattle, WA). Cells were tested for viability using Trypan Blue (Gibco BRL). Ten milliliters of Ficoll/Hypaque Plus (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) was layered under the cell suspension and spun for 5 30 minutes at 1800 rpm and allowed to stop with the brake off. The interface was then removed and transferred to a fresh 50 ml Falcon tube, brought up to a final volume of 40 ml with PBS and spun for 10 minutes at 1200 rpm with the brake on. The viability of the isolated cells was again tested using Trypan Blue. Alternately fresh drawn human blood was diluted 1:1 with PBS (Gibco BRL) and layered over Ficoll/Hypaque Plus 10 (Pharmacia), spun and washed as above. Cells isolated from either fresh or frozen sources gave equivalent results.

B cells were purified from the Ficoll floated peripheral blood cells of normal human donors (above) with anti-CD19 magnetic beads (Milenyi Biotec, Auburn, CA) following the manufacturer's instructions. The purity of the resulting 15 preparations was monitored by flow cytometric analysis with anti-CD22 FITC Ab (Pharmingen, SanDiego, CA). B cell preparations were typically >90% pure.

#### B. Purification of Murine B cells

A suspension of murine splenocytes was prepared by teasing adult 20 C57Bl/6 mouse (Charles River Laboratories, Wilmington, MA) spleens apart with bent needles in B cell medium. RBCs were removed by hypotonic lysis. CD43 positive cells were removed with CD43 magnetic beads (Milenyi Biotec) following the manufacturer's instructions. The purity of the resulting preparations was monitored by flow cytometric analysis with anti-CD45R FITC Ab (Pharmingen). B cell preparations 25 were typically >90% pure.

#### C. Proliferation of anti-CD40-stimulated B-Cells in the presence of human or murine IL-21

The B cells from either the human or mouse source were resuspended at 30 a final concentration of  $1 \times 10^6$  cells/ml in B cell medium and plated at 100  $\mu$ l/well in a 96 well U bottom plate (Falcon, VWR) containing various stimulation conditions to bring the final volume to 200  $\mu$ l/well. For anti-CD40 stimulation human cultures were supplemented with 1 $\mu$ g/ml anti-human CD40 (Genzyme, Cambridge, MA) and mouse cultures were supplemented with 1 $\mu$ g/ml anti-murine CD40 (Serotec, UK). Human or 35 murine IL-21 was added at dilutions ranging from 1 pg/ml-100 ng/ml. The specificity of the effect of IL-21 was confirmed by inhibition of IL-21 with 25mg/ml soluble human

IL-21 CEE. All treatments were performed in triplicate. The cells were then incubated at 37°C in a humidified incubator for 120 hours (human) or 72 hours (mouse). Sixteen hours prior to harvesting, 1  $\mu$ Ci  $^3$ H-thymidine (Amersham, Piscataway, NJ) was added to all wells to assess whether the B-cells had proliferated. The cells were harvested into  
5 a 96 well filter plate (UniFilter GF/C, Packard, Meriden, CT) using a cell harvester (Packard) and collected according to manufacturer's instructions. The plates were dried at 55°C for 20-30 minutes and the bottom of the wells were sealed with an opaque plate sealer. To each well was added 0.25 ml of scintillation fluid (Microscint-O, Packard) and the plate was read using a TopCount Microplate Scintillation Counter (Packard).

10 Incubation with IL-21 at concentrations of 3 ng/ml or more enhanced the proliferation induced by soluble anti-CD40 in a dose dependent manner in both murine and human B cells by as much as 30 fold. The murine and human B cells responded equally as well to their respective IL-21 species. In both species, the stimulation was specific to IL-21, as it was reversed by the presence of soluble IL-21 receptor in the  
15 culture.

#### D. Proliferation of anti-IgM-stimulated B-Cells in the presence of human or murine IL-21

The B cells from either human or mouse source as described above were  
20 plated as described above. For anti-IgM stimulation of human cells the plates were pre-coated overnight with 10mg/ml F(ab')<sub>2</sub> anti-human IgM Abs (Southern Biotech Associates, Birmingham, Alabama) and washed with sterile media just prior to use. The cultures were supplemented with 0-10 ng/ml hu rIL-4 (R&D Systems, Minneapolis, MN). For anti-IgM stimulation of murine cells soluble anti-IgM (Biosource, Camarillo,  
25 CA) was added to the cultures at 10 mg/ml. To each of the preceding anti-IgM/IL-4 conditions, human or murine IL-21 was added at dilutions ranging from 1 pg/ml-100 ng/ml as described above. The specificity of the effect of IL-21 was confirmed by inhibition with soluble human IL-21 receptor. All treatments were performed in triplicate. The cells were incubated, labeled with  $^3$ H-thymidine, harvested, and  
30 analyzed.

Incubation with IL-21 at concentrations of 0.3 ng/ml or more inhibited the proliferation induced by insoluble anti-IgM (mouse) or anti-IgM and IL-4 (human) in a dose-dependent manner. This inhibition was specific to IL-21, as it was reversed by the presence of soluble IL-21 receptor in the culture.

### Example 7

#### <sup>125</sup>I-labeled Human IL-21 Binding Study in Cell Lines

25 micrograms of purified human IL-21 was labeled with 2 mCi <sup>125</sup>I using IODO-BEADS® (Pierce, Rockford Illinois), according to manufacturer's instructions. This labeled protein was used to assess human IL-21 binding to human Raji cells (ATCC No. CCL-86), using binding to wild-type murine BaF3 cells, and BaF3 cells transfected with IL-21 receptor (BaF3/hIL-21 cells) as controls. IL-21 binding to BaF3/hIL-21 cells was expected (positive control), while no binding to wild-type BaF3 cells was expected (negative control), based on proliferation assay. About 5X10<sup>5</sup> Raji cells/well, 1X10<sup>6</sup> BaF3/hIL-21 and 1X10<sup>6</sup> BaF3 cells/well, were each plated in 96-well plates. Ten ng/ml of labeled human IL-21 was added in duplicate to wells, with a dilution series of unlabeled human IL-21 competitor added from 250 fold molar excess in 1:4 dilutions down to .061 fold molar excess. Each point was run in duplicate. After the labeled human IL-21 was added to wells, it was allowed to incubate at 4°C for 2 h to allow for binding of IL-21 to the cells. The cells were then washed 3X in binding buffer (RPMI-1710 (JRH Bioscience) with 1% BSA (Sigma)), and counted on the COBRA II AUTO-GAMMA gamma counter (Packard Instrument Company, Meriden, CT).

Binding of the labeled IL-21 to cells was evident in the Raji and the BaF3/hIL-21 receptor cells. In addition, for Raji cells, an average 250 fold molar excess of unlabeled IL-21 decreased binding 3 fold in the presence of a non-specific unlabeled competitor (Interferon Gamma from R&D Systems, Minneapolis, MN), and 3.7 fold relative to no competitor. Competition was observed in a dose dependent fashion for the specific unlabeled competitor, human IL-21. Thus, the IL-21 binding to Raji cells was specific. Similarly, for positive control BaF3/IL-21 receptor cells, the 250 fold molar excess of unlabeled IL-21 decreased binding 2 fold relative to the non-specific competitor and 3.06 fold relative to no competitor. Thus, the IL-21 binding to BaF3/IL-21 receptor cells also was specific. No comparable binding was observed with the wild-type BaF3 cells. Thus, the IL-21 was shown to bind specifically to Raji cells, and to BaF3/hIL-21 cells, but not to the negative control BaF3 cells.

### Example 8

#### IL-21 Receptor Expression On Human Blood Cells

##### A. Preparation and Culture of Human Peripheral Blood Cells

Fresh drawn human blood was diluted 1:1 with PBS (GIBCO BRL) and layered over Ficoll/Hypaque Plus (Pharmacia LKB Biotechnology Inc., Piscataway, NJ)

and spun for 30 minutes at 1800 rpm and allowed to stop with the brake off. The interface layer was removed and transferred to a fresh 50 ml Falcon tube (Falcon, VWR, Seattle, WA), brought up to a final volume of 40 ml with PBS and spun for 10 minutes at 1200 rpm with the brake on. The viability of the isolated cells was tested using Trypan Blue (GIBCO BRL) and the cells were resuspended at a final concentration of  $1 \times 10^6$  cells/ml cell medium (RPMI Medium 1640, 10% Heat inactivated fetal bovine serum, 5% L-glutamine, 5% Pen/Strep) (GIBCO BRL).

Cells were cultured in 6 well plates (Falcon, VWR) for 0, 4 or 24 hours with a variety of different stimuli described below. Anti-IgM, anti-CD40 and anti-CD3 stimulation were done as in Example 44 and Example 42. Phorbol myristate acetate (PMA) and ionomycin (Sigma, St. Louis, MO) (Example 5C) were added to appropriate wells at 10 ng/ml and 0.5 mg/ml respectively. The cells were incubated at 37°C in a humidified incubator for various times.

#### B. Antibody Staining and Analysis

Cells were collected out of the plates, washed and resuspended in ice cold staining media (HBSS, 1% fetal bovine serum, 0.1% sodium azide) at a concentration of about ten million cells per milliliter. Blocking of Fc receptor and non-specific binding of antibodies to the cells was achieved by adding 10% normal goat serum (Gemini Bioproducts, Woodland, CA) and 10% normal human serum (Ultraserum, Gemini) to the cell suspension. Aliquots of the cell suspensions were mixed with a FITC labeled monoclonal antibody against one of the lineage markers CD3, CD19 or CD14 (PharMingen, La Jolla, CA) and a biotinylated monoclonal antibody against the human IL-21 receptor. Staining specificity was determined by competition using IL-21CEE soluble receptor at a ten fold mass excess. After incubation on ice for 60 minutes the cells were washed twice with ice cold staining media and resuspended in 50 ml staining media containing streptavidin-PE (Caltag, Burlingame, CA). After a 30 minute incubation on ice, the cells were washed twice with ice cold wash buffer (PBS, 1% fetal bovine serum, 0.1% sodium azide) and resuspended in wash buffer containing 1mg/ml 7-AAD (Molecular Probes, Eugene, OR) as a viability marker. Flow data was acquired on living cells using a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA). Both acquisition and analysis were performed using CellQuest software (BD Immunocytometry Systems).

Results of staining by anti-IL-21 antibody showed that the human IL-21 receptor is expressed on human peripheral blood cells expressing either CD3, CD19 or CD14. Staining on CD3 and CD19 cells was specific, as evidenced by absolute



competition with the IL-21 soluble receptor. Staining on CD14 cells showed some specificity for the Ligand, as evidenced by partial competition with the soluble receptor. Activation of either T cells with anti-CD3 or B cells with anti-CD40 resulted in an increased level of cell surface IL-21 receptor at 24 hours. No increase in the level of expression of IL-21 receptor was seen at 4 hours with any stimulus on either cell population. Treatment of the cells with IL-21 resulted in a decrease of IL-21 receptor staining on CD3 positive and CD19 positive cells but not CD14 positive cells at both 4 and 24 hours.

### Example 9

#### Preparation of IL-21 Antagonists

##### Construction of IL-21 Mutants

A total of seven constructs (of which three are shown herein) were made using a Stratagene QuikChange Mutagenesis kit (Catalog 200518). 100 ng. of template was used with 125 ng. of each of the sense and antisense oligonucleotides. 25 cycles each of 94°C for 1 min, 55°C for 1 min. and 68°C for 25 min. were performed for each separate reaction. The following oligonucleotide and template pairs were used:

Mutations were incorporated into the IL-21 coding sequence included in the baculovirus expression vector BVpIL-21 using site directed mutagenesis. 100 ng of template was combined with 125 ng each of antisense and sense oligonucleotides as shown in the table below. Thermally stable DNA polymerase was added and DNA containing the desired mutations was synthesized during 25 thermal cycles of 94°C for 1 minute, 55°C for 1 minute and 68°C for 25 minutes.

<u>Construct</u>	<u>Oligos(sense/antisense)</u>	<u>Template</u>
I148Stop	zc27885/zc27884 (SEQ ID. NOS: 25 and 26)	BV palpha11L
Q145D/I148D	zc37198/zc37199 (SEQ ID. NOS: 27 and 28)	BV palpha11L-Q145D
Q145D/I148Stop	zc37200/zc37203 (SEQ ID. NOS: 29 and 30)	BV palpha11L-Q145D

Following synthesis the parental, non-mutated DNA was removed by digestion with the restriction enzyme DPN1, and the mutated DNA was transformed into ElectroCompetent DH10B (Life Technologies). The transformed cells were plated for selection onto LB plates containing 100 µg/mL ampicillin. Clones were analyzed by DNA sequencing and 1 µl of the positive clone was transformed into 20 µl DH10Bac Max Efficiency competent cells (Life Technologies) according to manufacturer's instructions.

The resulting mutants were designated Q153D;I156D as shown in SEQ ID NO: 5 (nucleotide sequence) and SEQ ID NO: 6 (amino acid sequence), and I156ST;Q153D as shown in SEQ ID NO: 3 (nucleotide sequence), and SEQ ID NO: 4 (amino acid sequence.)

5

#### Example 10

##### A. Identification of Antagonist Activity in IL-21 Mutants

10 IL-21 mutant clones were expressed in baculovirus as described in Example 5. Insect cells expressing wild type human IL-21 and each of the D-helix mutants was cultured in serum free conditions. Culture media was collected and the concentration of IL-21 or mutant IL-21 was determined by western blot analysis using anti-IL-21 rabbit polyclonal antibody, D1048, for detection.

15

##### B. Human IL-21 binding studies in cell lines

Each mutant protein was evaluated for its ability to inhibit binding of  $^{125}\text{I}$ -IL-21 to receptors expressed on the surface of BHK cells transfected with IL-21 receptor and IL-2R $\gamma$ . An  $\text{IC}_{50}$  for inhibition of  $^{125}\text{I}$ -IL-21 binding was determined for each protein. Receptor activation by each protein is expressed as an  $\text{EC}_{50}$  determined by the ability to support the growth of a IL-21 dependent BaF3 cell line expressing IL-21R $\alpha$  and IL2R $\gamma$ .

25 Human IL-21 was iodinated using IODO-BEADS® (Pierce) according to manufacturer's directions. For competition binding assays, 250 pM  $^{125}\text{I}$ -IL-21 was used increasing concentrations of competitor proteins. Cells, in 24 well tissue culture dishes, were incubated in 250  $\mu\text{l}$  of binding buffer (RPMI1640 (GIBCO-BRL), 20 mM HEPES, pH7.4, 1mg/mL BSA (Sigma)) containing  $^{125}\text{I}$ -IL-21, with or without inhibitors, for 2 hours at 4°C. Unbound ligand was removed by by three washes with ice cold binding buffer. The cells were then extracted with PBS, 1% Triton-X100 (Sigma) and the extracts counted in a gamma counter (Packard). Analysis was done using GraphPad PRISM® (GraphPad software, Inc., San Diego, CA)

35 Transfected BaF3 cells expressing human IL-21 receptor are washed 3X with RPMI/10%FBS to remove trace amounts of IL-3. BaF3 cells are then plated at 7500 cells/well in 96 well dish and cultured for three days in the absence or presence of IL-21 or IL-21 mutants at concentrations ranging from 1 ng/mL to 1  $\mu\text{g/mL}$ . After three days the cultures are assayed for viable cells using AlamarBlue (Alamar, Inc.)

according to manufacturer's instructions. EC50's for IL-21 and IL-21 mutants are calculated using GraphPad PRISM® (GraphPad software, Inc.).

5

Table 6

<u>Sample</u>	<u>IC<sub>50</sub> (nM)</u>	<u>EC<sub>50</sub> (nM)</u>
hIL-21	10	10
10 I156STOP	20	100
Q153D;I156D	10	ND
I156ST;Q153D	10	ND

IC<sub>50</sub> and EC<sub>50</sub> are expressed as nM and ND represents no activity detected.

15

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

We claim:

1. An isolated polynucleotide molecule comprising a nucleotide sequence as shown in SEQ ID NO: 3 or SEQ ID NO: 5.
2. An isolated polynucleotide molecule encoding an IL-21 antagonist polypeptide comprising an amino acid sequence as shown as in SEQ ID NO: 4 or SEQ ID NO: 6.
3. An isolated polypeptide comprising an amino acid sequence as shown in SEQ ID NO: 4 or SEQ ID NO: 6.
4. A fusion protein comprising at least two polypeptides, wherein at least one of the polypeptides comprises a polypeptide selected from the group consisting of an amino acid sequence as shown in SEQ ID NO:4 or SEQ ID NO:6, and a second polypeptide sequence.
5. An expression vector, comprising the isolated nucleic acid molecule of claim 1, a transcription promoter, and a transcription terminator, wherein the promoter is operably linked with the nucleic acid molecule, and wherein the nucleic acid molecule is operably linked with the transcription terminator.
6. A recombinant host cell comprising the expression vector of claim 5, wherein the host cell is selected from the group consisting of bacterium, yeast cell, fungal cell, insect cell, mammalian cell, and plant cell.
7. A method of producing a polypeptide, the method comprising the step of culturing recombinant host cells that comprise the expression vector of claim 5, and that produce the polypeptide.

## SEQUENCE LISTING

&lt;110&gt; ZymoGenetics, Inc.

&lt;120&gt; ZALPHA11 LIGAND ANTAGONISTS

&lt;130&gt; 01-37PC

&lt;150&gt; 60/337,586

&lt;151&gt; 2001-11-05

&lt;160&gt; 30

&lt;170&gt; FastSEQ for Windows Version 4.0

&lt;210&gt; 1

&lt;211&gt; 642

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (47)...(532)

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 Ser Pro Gly Asn Met Glu Arg Ile Val Ile Cys Leu Met Val Ile Phe  
 5 10 15

ttg ggg aca ctg gtc cac aaa tca agc tcc caa ggt caa gat cgc cac 151  
 Leu Gly Thr Leu Val His Lys Ser Ser Ser Gln Gly Gln Asp Arg His  
 20 25 30 35

atg att aga atg cgt caa ctt ata gat att gtt gat cag ctg aaa aat 199  
 Met Ile Arg Met Arg Gln Leu Ile Asp Ile Val Asp Gln Leu Lys Asn  
 40 45 50

tat gtg aat gac ttg gtc cct gaa ttt ctg cca gct cca gaa gat gta 247  
Tyr Val Asn Asp Leu Val Pro Glu Phe Leu Pro Ala Pro Glu Asp Val  
55 60 65

gag aca aac tgt gag tgg tca gct ttt tcc tgt ttt cag aag gcc caa 295  
Glu Thr Asn Cys Glu Trp Ser Ala Phe Ser Cys Phe Gln Lys Ala Gln  
70 75 80

cta aag tca gca aat aca gga aac aat gaa agg ata atc aat gta tca 343  
Leu Lys Ser Ala Asn Thr Gly Asn Asn Glu Arg Ile Ile Asn Val Ser  
85 90 95

att aaa aag ctg aag agg aaa cca cct tcc aca aat gca ggg aga aga 391  
Ile Lys Lys Leu Lys Arg Lys Pro Pro Ser Thr Asn Ala Gly Arg Arg  
100 105 110 115

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Gln Lys His Arg Leu Thr Cys Pro Ser Cys Asp Ser Tyr Glu Lys Lys  
120 125 130

cca ccc aaa gaa ttc cta gaa aga ttc aaa tca ctt ctc caa aag atg 487  
Pro Pro Lys Glu Phe Leu Glu Arg Phe Lys Ser Leu Leu Gln Lys Met  
135 140 145

att cat cag cat ctg tcc tct aga aca cac gga agt gaa gat tcc 532  
Ile His Gln His Leu Ser Ser Arg Thr His Gly Ser Glu Asp Ser  
150 155 160

tgaggatcta acttgcagtt ggacactatg ttacatactc taatatagta gtgaaagtca 592  
tttcttttga ttccaaqtgq aggaqcccta ttaaattata taaagaaata 642

<210> 2

<211> 162

&lt;212&gt; PRT

<213> Homo sapiens

<400> 2

Met Arg Ser Ser Pro Gly Asn Met Glu Arg Ile Val Ile Cys Leu Met  
1 5 10 15  
Val Ile Phe Leu Gly Thr Leu Val His Lys Ser Ser Ser Gln Gly Gln  
20 25 30  
Asp Arg His Met Ile Arg Met Arg Gln Leu Ile Asp Ile Val Asp Gln  
35 40 45

Leu Lys Asn Tyr Val Asn Asp Leu Val Pro Glu Phe Leu Pro Ala Pro  
 50 55 60  
 Glu Asp Val Glu Thr Asn Cys Glu Trp Ser Ala Phe Ser Cys Phe Gln  
 65 70 75 80  
 Lys Ala Gln Leu Lys Ser Ala Asn Thr Gly Asn Asn Glu Arg Ile Ile  
 85 90 95  
 Asn Val Ser Ile Lys Lys Leu Lys Arg Lys Pro Pro Ser Thr Asn Ala  
 100 105 110  
 Gly Arg Arg Gln Lys His Arg Leu Thr Cys Pro Ser Cys Asp Ser Tyr  
 115 120 125  
 Glu Lys Lys Pro Pro Lys Glu Phe Leu Glu Arg Phe Lys Ser Leu Leu  
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 Gln Lys Met Ile His Gln His Leu Ser Ser Arg Thr His Gly Ser Glu  
 145 150 155 160  
 Asp Ser

&lt;210&gt; 3

&lt;211&gt; 444

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; zalpha11 ligand Q153ST/I156D

&lt;221&gt; CDS

&lt;222&gt; (1)...(444)

&lt;400&gt; 3

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 Met Asp Ser Ser Pro Gly Asn Met Glu Arg Ile Val Ile Cys Leu Met  
 1 5 10 15

gtc atc ttc ttg ggg aca ctg gtc cac aaa tca agc tcc caa ggt caa 96  
 Val Ile Phe Leu Gly Thr Leu Val His Lys Ser Ser Ser Gln Gly Gln  
 20 25 30

gat cgc cac atg att aga atg cgt caa ctt ata gat att gtt gat cag 144  
 Asp Arg His Met Ile Arg Met Arg Gln Leu Ile Asp Ile Val Asp Gln  
 35 40 45

ctg aaa aat tat gtg aat gac ttg gtc cct gaa ttt ctg cca gct cca 192  
Leu Lys Asn Tyr Val Asn Asp Leu Val Pro Glu Phe Leu Pro Ala Pro  
50 55 60

gaa gat gta gag aca aac tgt gag tgg tca gct ttt tcc tgt ttt cag 240  
Glu Asp Val Glu Thr Asn Cys Glu Trp Ser Ala Phe Ser Cys Phe Gln  
65 70 75 80

aag gcc caa cta aag tca gca aat aca gga aac aat gaa agg ata atc 288  
Lys Ala Gln Leu Lys Ser Ala Asn Thr Gly Asn Asn Glu Arg Ile Ile  
85 90 95

aat gta tca att aaa aag ctg aag agg aaa cca cct tcc aca aat gca 336  
Asn Val Ser Ile Lys Lys Leu Lys Arg Lys Pro Pro Ser Thr Asn Ala  
100 105 110

ggg aga aga cag aaa cac aga cta aca tgc cct tca tgt gat tct tat 384  
Gly Arg Arg Gln Lys His Arg Leu Thr Cys Pro Ser Cys Asp Ser Tyr  
115 120 125

gag aaa aaa cca ccc aaa gaa ttc cta gaa aga ttc aaa tca ctt ctc 432  
Glu Lys Lys Pro Pro Lys Glu Phe Leu Glu Arg Phe Lys Ser Leu Leu  
130 135 140

gat aag atg taa 444  
Asp Lys Met \*  
145

<210> 4

&lt;211&gt; 147

<212> PRT

### <213> Artificial Sequence

 $\langle 220 \rangle$ 

<223> zalpha11 ligand Q153ST/I156D

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Val Ile Phe Leu Gly Thr Leu Val His Lys Ser Ser Ser Gln Gly Gln  
20 25 30



Asp Arg His Met Ile Arg Met Arg Gln Leu Ile Asp Ile Val Asp Gln  
           35                    40                    45  
 Leu Lys Asn Tyr Val Asn Asp Leu Val Pro Glu Phe Leu Pro Ala Pro  
           50                    55                    60  
 Glu Asp Val Glu Thr Asn Cys Glu Trp Ser Ala Phe Ser Cys Phe Gln  
   65                    70                    75                    80  
 Lys Ala Gln Leu Lys Ser Ala Asn Thr Gly Asn Asn Glu Arg Ile Ile  
                     85                    90                    95  
 Asn Val Ser Ile Lys Lys Leu Lys Arg Lys Pro Pro Ser Thr Asn Ala  
                     100                    105                    110  
 Gly Arg Arg Gln Lys His Arg Leu Thr Cys Pro Ser Cys Asp Ser Tyr  
           115                    120                    125  
 Glu Lys Lys Pro Pro Lys Glu Phe Leu Glu Arg Phe Lys Ser Leu Leu  
           130                    135                    140  
 Asp Lys Met  
 145

&lt;210&gt; 5

&lt;211&gt; 489

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; zalpha11 ligand Q153D/I156D

&lt;221&gt; CDS

&lt;222&gt; (1)...(489)

&lt;400&gt; 5

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   1                    5                    10                    15

gtc atc ttc ttg ggg aca ctg gtc cac aaa tca agc tcc caa ggt caa 96  
 Val Ile Phe Leu Gly Thr Leu Val His Lys Ser Ser Ser Gln Gly Gln  
           20                    25                    30

gat cgc cac atg att aga atg cgt caa ctt ata gat att gtt gat cag 144  
 Asp Arg His Met Ile Arg Met Arg Gln Leu Ile Asp Ile Val Asp Gln  
           35                    40                    45

ctg aaa aat tat gtg aat gac ttg gtc cct gaa ttt ctg cca gct cca 192  
 Leu Lys Asn Tyr Val Asn Asp Leu Val Pro Glu Phe Leu Pro Ala Pro  
           50                          55                          60

gaa gat gta gag aca aac tgt gag tgg tca gct ttt tcc tgt ttt cag 240  
 Glu Asp Val Glu Thr Asn Cys Glu Trp Ser Ala Phe Ser Cys Phe Gln  
           65                          70                          75                          80

aag gcc caa cta aag tca gca aat aca gga aac aat gaa agg ata atc 288  
 Lys Ala Gln Leu Lys Ser Ala Asn Thr Gly Asn Asn Glu Arg Ile Ile  
                           85                          90                          95

aat gta tca att aaa aag ctg aag agg aaa cca cct tcc aca aat gca 336  
 Asn Val Ser Ile Lys Lys Leu Lys Arg Lys Pro Pro Ser Thr Asn Ala  
                           100                          105                          110

ggg aga aga cag aaa cac aga cta aca tgc cct tca tgt gat tct tat 384  
 Gly Arg Arg Gln Lys His Arg Leu Thr Cys Pro Ser Cys Asp Ser Tyr  
           115                          120                          125

gag aaa aaa cca ccc aaa gaa ttc cta gaa aga ttc aaa tca ctt ctc 432  
 Glu Lys Lys Pro Pro Lys Glu Phe Leu Glu Arg Phe Lys Ser Leu Leu  
           130                          135                          140

gat aag atg gat cat cag cat ctg tcc tct aga aca cac gga agt gaa 480  
 Asp Lys Met Asp His Gln His Leu Ser Ser Arg Thr His Gly Ser Glu  
           145                          150                          155                          160

gat tcc tga 489  
 Asp Ser \*

<210> 6

<211> 162

<212> PRT

<213> Artificial Sequence

<220>

<223> zalpha11 ligand Q153D/I156D

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Met Asp Ser Ser Pro Gly Asn Met Glu Arg Ile Val Ile Cys Leu Met  
 1 5 10 15  
 Val Ile Phe Leu Gly Thr Leu Val His Lys Ser Ser Ser Gln Gly Gln  
 20 25 30  
 Asp Arg His Met Ile Arg Met Arg Gln Leu Ile Asp Ile Val Asp Gln  
 35 40 45  
 Leu Lys Asn Tyr Val Asn Asp Leu Val Pro Glu Phe Leu Pro Ala Pro  
 50 55 60  
 Glu Asp Val Glu Thr Asn Cys Glu Trp Ser Ala Phe Ser Cys Phe Gln  
 65 70 75 80  
 Lys Ala Gln Leu Lys Ser Ala Asn Thr Gly Asn Asn Glu Arg Ile Ile  
 85 90 95  
 Asn Val Ser Ile Lys Lys Leu Lys Arg Lys Pro Pro Ser Thr Asn Ala  
 100 105 110  
 Gly Arg Arg Gln Lys His Arg Leu Thr Cys Pro Ser Cys Asp Ser Tyr  
 115 120 125  
 Glu Lys Lys Pro Pro Lys Glu Phe Leu Glu Arg Phe Lys Ser Leu Leu  
 130 135 140  
 Asp Lys Met Asp His Gln His Leu Ser Ser Arg Thr His Gly Ser Glu  
 145 150 155 160  
 Asp Ser

&lt;210&gt; 7

&lt;211&gt; 153

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 7

Met Tyr Arg Met Gln Leu Leu Ser Cys Ile Ala Leu Ser Leu Ala Leu  
 1 5 10 15  
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 20 25 30  
 Gln Leu Glu His Leu Leu Leu Asp Leu Gln Met Ile Leu Asn Gly Ile  
 35 40 45  
 Asn Asn Tyr Lys Asn Pro Lys Leu Thr Arg Met Leu Thr Phe Lys Phe  
 50 55 60  
 Tyr Met Pro Lys Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu  
 65 70 75 80  
 Glu Glu Leu Lys Pro Leu Glu Glu Val Leu Asn Leu Ala Gln Ser Lys  
 85 90 95

Asn Phe His Leu Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn Val Ile  
                   100                  105                  110  
 Val Leu Glu Leu Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala  
                   115                  120                  125  
 Asp Glu Thr Ala Thr Ile Val Glu Phe Leu Asn Arg Trp Ile Thr Phe  
                   130                  135                  140  
 Cys Gln Ser Ile Ile Ser Thr Leu Thr  
                   145                  150

&lt;210&gt; 8

&lt;211&gt; 153

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 8

Met Gly Leu Thr Ser Gln Leu Leu Pro Pro Leu Phe Phe Leu Leu Ala  
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 Cys Ala Gly Asn Phe Val His Gly His Lys Cys Asp Ile Thr Leu Gln  
                   20                  25                  30  
 Glu Ile Ile Lys Thr Leu Asn Ser Leu Thr Glu Gln Lys Thr Leu Cys  
                   35                  40                  45  
 Thr Glu Leu Thr Val Thr Asp Ile Phe Ala Ala Ser Lys Asn Thr Thr  
                   50                  55                  60  
 Glu Lys Glu Thr Phe Cys Arg Ala Ala Thr Val Leu Arg Gln Phe Tyr  
   65                  70                  75                  80  
 Ser His His Glu Lys Asp Thr Arg Cys Leu Gly Ala Thr Ala Gln Gln  
                   85                  90                  95  
 Phe His Arg His Lys Gln Leu Ile Arg Phe Leu Lys Arg Leu Asp Arg  
                   100                  105                  110  
 Asn Leu Trp Gly Leu Ala Gly Leu Asn Ser Cys Pro Val Lys Glu Ala  
                   115                  120                  125  
 Asn Gln Ser Thr Leu Glu Asn Phe Leu Glu Arg Leu Lys Thr Ile Met  
                   130                  135                  140  
 Arg Glu Lys Tyr Ser Lys Cys Ser Ser  
                   145                  150

&lt;210&gt; 9

&lt;211&gt; 162

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 9

```

Met Arg Ile Ser Lys Pro His Leu Arg Ser Ile Ser Ile Gln Cys Tyr
 1           5           10           15
Leu Cys Leu Leu Leu Asn Ser His Phe Leu Thr Glu Ala Gly Ile His
          20           25           30
Val Phe Ile Leu Gly Cys Phe Ser Ala Gly Leu Pro Lys Thr Glu Ala
        35           40           45
Asn Trp Val Asn Val Ile Ser Asp Leu Lys Lys Ile Glu Asp Leu Ile
        50           55           60
Gln Ser Met His Ile Asp Ala Thr Leu Tyr Thr Glu Ser Asp Val His
65           70           75           80
Pro Ser Cys Lys Val Thr Ala Met Lys Cys Phe Leu Leu Glu Leu Gln
          85           90           95
Val Ile Ser Leu Glu Ser Gly Asp Ala Ser Ile His Asp Thr Val Glu
        100          105          110
Asn Leu Ile Ile Leu Ala Asn Asn Ser Leu Ser Ser Asn Gly Asn Val
        115          120          125
Thr Glu Ser Gly Cys Lys Glu Cys Glu Glu Leu Glu Glu Lys Asn Ile
        130          135          140
Lys Glu Phe Leu Gln Ser Phe Val His Ile Val Gln Met Phe Ile Asn
145          150          155          160
Thr Ser

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&lt;210&gt; 10

&lt;211&gt; 144

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 10

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Met Trp Leu Gln Ser Leu Leu Leu Leu Gly Thr Val Ala Cys Ser Ile
 1           5           10           15
Ser Ala Pro Ala Arg Ser Pro Ser Pro Ser Thr Gln Pro Trp Glu His
          20           25           30
Val Asn Ala Ile Gln Glu Ala Arg Arg Leu Leu Asn Leu Ser Arg Asp
        35           40           45
Thr Ala Ala Glu Met Asn Glu Thr Val Glu Val Ile Ser Glu Met Phe
        50           55           60
Asp Leu Gln Glu Pro Thr Cys Leu Gln Thr Arg Leu Glu Leu Tyr Lys
65           70           75           80
Gln Gly Leu Arg Gly Ser Leu Thr Lys Leu Lys Gly Pro Leu Thr Met
          85           90           95

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Met	Ala	Ser	His	Tyr	Lys	Gln	His	Cys	Pro	Pro	Thr	Pro	Glu	Thr	Ser
		100						105					110		
Cys	Ala	Thr	Gln	Ile	Ile	Thr	Phe	Glu	Ser	Phe	Lys	Glu	Asn	Leu	Lys
		115				120					125				
Asp	Phe	Leu	Leu	Val	Ile	Pro	Phe	Asp	Cys	Trp	Glu	Pro	Val	Gln	Glu
	130					135					140				

&lt;210&gt; 11

&lt;211&gt; 33

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; oligonucleotide primer ZC17,212

&lt;400&gt; 11

ggggaattcg aagccatgcc ctcttgggcc etc

33

&lt;210&gt; 12

&lt;211&gt; 30

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; oligonucleotide primer ZC19,914

&lt;400&gt; 12

caatggatgg gtctttagca gcagtaggcc

30

&lt;210&gt; 13

&lt;211&gt; 30

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; oligonucleotide primer ZC19,913

&lt;400&gt; 13

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30

&lt;210&gt; 14

&lt;211&gt; 33

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; oligonucleotide primer ZC20.097

&lt;400&gt; 14

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33

&lt;210&gt; 15

&lt;211&gt; 1614

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)...(1614)

&lt;400&gt; 15

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Met Pro Arg Gly Trp Ala Ala Pro Leu Leu Leu Leu Leu Gln Gly

1

5

10

15

ggc tgg ggc tgc ccc gac ctc gtc tgc tac acc gat tac ctc cag acg 96

Gly Trp Gly Cys Pro Asp Leu Val Cys Tyr Thr Asp Tyr Leu Gln Thr

20

25

30

gtc atc tgc atc ctg gaa atg tgg aac ctc cac ccc agc acg ctc acc 144

Val Ile Cys Ile Leu Glu Met Trp Asn Leu His Pro Ser Thr Leu Thr

35

40

45

ctt acc tgg caa gac cag tat gaa gag ctg aag gac gag gcc acc tcc 192

Leu Thr Trp Gln Asp Gln Tyr Glu Glu Leu Lys Asp Glu Ala Thr Ser

50

55

60

tgc agc ctc cac agg tcg gcc cac aat gcc acg cat gcc acc tac acc 240

Cys Ser Leu His Arg Ser Ala His Asn Ala Thr His Ala Thr Tyr Thr

65

70

75

80

tgc cac atg gat gta ttc cac ttc atg gcc gac gac att ttc agt gtc 288

Cys His Met Asp Val Phe His Phe Met Ala Asp Asp Ile Phe Ser Val

85

90

95

aac atc aca gac cag tct ggc aac tac tcc cag gag tgt ggc agc ttt	336
Asn Ile Thr Asp Gln Ser Gly Asn Tyr Ser Gln Glu Cys Gly Ser Phe	
100 105 110	
ctc ctg gct gag agc atc aag ccg gct ccc cct ttc aac gtg act gtg	384
Leu Leu Ala Glu Ser Ile Lys Pro Ala Pro Phe Asn Val Thr Val	
115 120 125	
acc ttc tca gga cag tat aat atc tcc tgg cgc tca gat tac gaa gac	432
Thr Phe Ser Gly Gln Tyr Asn Ile Ser Trp Arg Ser Asp Tyr Glu Asp	
130 135 140	
cct gcc ttc tac atg ctg aag ggc aag ctt cag tat gag ctg cag tac	480
Pro Ala Phe Tyr Met Leu Lys Gly Lys Leu Gln Tyr Glu Leu Gln Tyr	
145 150 155 160	
agg aac cgg gga gac ccc tgg gct gtg agt ccg agg aga aag ctg atc	528
Arg Asn Arg Gly Asp Pro Trp Ala Val Ser Pro Arg Arg Lys Leu Ile	
165 170 175	
tca gtg gac tca aga agt gtc tcc ctc ctc ccc ctg gag ttc cgc aaa	576
Ser Val Asp Ser Arg Ser Val Ser Leu Leu Pro Leu Glu Phe Arg Lys	
180 185 190	
gac tcg agc tat gag ctg cag gtg ccg gca ggg ccc atg cct ggc tcc	624
Asp Ser Ser Tyr Glu Leu Gln Val Arg Ala Gly Pro Met Pro Gly Ser	
195 200 205	
tcc tac cag ggg acc tgg agt gaa tgg agt gac ccg gtc atc ttt cag	672
Ser Tyr Gln Gly Thr Trp Ser Glu Trp Ser Asp Pro Val Ile Phe Gln	
210 215 220	
acc cag tca gag gag tta aag gaa ggc tgg aac cct cac ctg ctg ctt	720
Thr Gln Ser Glu Glu Leu Lys Glu Gly Trp Asn Pro His Leu Leu Leu	
225 230 235 240	
ctc ctc ctg ctt gtc ata gtc ttc att cct gcc ttc tgg agc ctg aag	768
Leu Leu Leu Leu Val Ile Val Phe Ile Pro Ala Phe Trp Ser Leu Lys	
245 250 255	
acc cat cca ttg tgg agg cta tgg aag aag ata tgg gcc gtc ccc agc	816
Thr His Pro Leu Trp Arg Leu Trp Lys Lys Ile Trp Ala Val Pro Ser	
260 265 270	



cct gag cgg ttc ttc atg ccc ctg tac aag ggc tgc agc gga gac ttc	864
Pro Glu Arg Phe Phe Met Pro Leu Tyr Lys Gly Cys Ser Gly Asp Phe	
275 280 285	
aag aaa tgg gtg ggt gca ccc ttc act ggc tcc agc ctg gag ctg gga	912
Lys Lys Trp Val Gly Ala Pro Phe Thr Gly Ser Ser Leu Glu Leu Gly	
290 295 300	
ccc tgg agc cca gag gtg ccc tcc acc ctg gag gtg tac agc tgc cac	960
Pro Trp Ser Pro Glu Val Pro Ser Thr Leu Glu Val Tyr Ser Cys His	
305 310 315 320	
cca cca cgg agc ccg gcc aag agg ctg cag ctc acg gag cta caa gaa	1008
Pro Pro Arg Ser Pro Ala Lys Arg Leu Gln Leu Thr Glu Leu Gln Glu	
325 330 335	
cca gca gag ctg gtg gag tct gac ggt gtg ccc aag ccc agc ttc tgg	1056
Pro Ala Glu Leu Val Glu Ser Asp Gly Val Pro Lys Pro Ser Phe Trp	
340 345 350	
ccg aca gcc cag aac tcg ggg ggc tca gct tac agt gag gag agg gat	1104
Pro Thr Ala Gln Asn Ser Gly Gly Ser Ala Tyr Ser Glu Glu Arg Asp	
355 360 365	
cgg cca tac ggc ctg gtg tcc att gac aca gtg act gtg cta gat gca	1152
Arg Pro Tyr Gly Leu Val Ser Ile Asp Thr Val Thr Val Leu Asp Ala	
370 375 380	
gag ggg cca tgc acc tgg ccc tgc agc tgt gag gat gac ggc tac cca	1200
Glu Gly Pro Cys Thr Trp Pro Cys Ser Cys Glu Asp Asp Gly Tyr Pro	
385 390 395 400	
gcc ctg gac ctg gat gct ggc ctg gag ccc agc cca ggc cta gag gac	1248
Ala Leu Asp Leu Asp Ala Gly Leu Glu Pro Ser Pro Gly Leu Glu Asp	
405 410 415	
cca ctc ttg gat gca ggg acc aca gtc ctg tcc tgt ggc tgt gtc tca	1296
Pro Leu Leu Asp Ala Gly Thr Thr Val Leu Ser Cys Gly Cys Val Ser	
420 425 430	

14

gct ggc agc cct ggg cta gga ggg ccc ctg gga agc ctc ctg gac aga 1344  
Ala Gly Ser Pro Gly Leu Gly Gly Pro Leu Gly Ser Leu Leu Asp Arg  
435 440 445

cta aag cca ccc ctt gca gat ggg gag gac tgg gct ggg gga ctg ccc 1392  
Leu Lys Pro Pro Leu Ala Asp Gly Glu Asp Trp Ala Gly Gly Leu Pro  
450 455 460

tgg ggt ggc cgg tca cct gga ggg gtc tca gag agt gag gcg ggc tca 1440  
Trp Gly Gly Arg Ser Pro Gly Gly Val Ser Glu Ser Glu Ala Gly Ser  
465 470 475 480

ccc ctg gcc ggc ctg gat atg gac acg ttt gac agt ggc ttt gtg ggc 1488  
Pro Leu Ala Gly Leu Asp Met Asp Thr Phe Asp Ser Gly Phe Val Gly  
485 490 495

tct gac tgc agc agc cct gtg gag tgt gac ttc acc agc ccc ggg gac 1536  
Ser Asp Cys Ser Ser Pro Val Glu Cys Asp Phe Thr Ser Pro Gly Asp  
500 505 510

gaa gga ccc ccc cgg agc tac ctc cgc cag tgg gtg gtc att cct ccg 1584  
Glu Gly Pro Pro Arg Ser Tyr Leu Arg Gln Trp Val Val Ile Pro Pro  
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cca ctt tcg agc cct gga ccc cag gcc agc 1614  
Pro Leu Ser Ser Pro Gly Pro Gln Ala Ser  
530 535

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<212> PRT

<213> Homo sapiens

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Met Pro Arg Gly Trp Ala Ala Pro Leu Leu Leu Leu Leu Gln Gly  
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Gly Trp Gly Cys Pro Asp Leu Val Cys Tyr Thr Asp Tyr Leu Gln Thr  
20 25 30  
Val Ile Cys Ile Leu Glu Met Trp Asn Leu His Pro Ser Thr Leu Thr  
35 40 45  
Leu Thr Trp Gln Asp Gln Tyr Glu Glu Leu Lys Asp Glu Ala Thr Ser  
50 55 60

Cys Ser Leu His Arg Ser Ala His Asn Ala Thr His Ala Thr Tyr Thr  
 65 70 75 80  
 Cys His Met Asp Val Phe His Phe Met Ala Asp Asp Ile Phe Ser Val  
 85 90 95  
 Asn Ile Thr Asp Gln Ser Gly Asn Tyr Ser Gln Glu Cys Gly Ser Phe  
 100 105 110  
 Leu Leu Ala Glu Ser Ile Lys Pro Ala Pro Pro Phe Asn Val Thr Val  
 115 120 125  
 Thr Phe Ser Gly Gln Tyr Asn Ile Ser Trp Arg Ser Asp Tyr Glu Asp  
 130 135 140  
 Pro Ala Phe Tyr Met Leu Lys Gly Lys Leu Gln Tyr Glu Leu Gln Tyr  
 145 150 155 160  
 Arg Asn Arg Gly Asp Pro Trp Ala Val Ser Pro Arg Arg Lys Leu Ile  
 165 170 175  
 Ser Val Asp Ser Arg Ser Val Ser Leu Leu Pro Leu Glu Phe Arg Lys  
 180 185 190  
 Asp Ser Ser Tyr Glu Leu Gln Val Arg Ala Gly Pro Met Pro Gly Ser  
 195 200 205  
 Ser Tyr Gln Gly Thr Trp Ser Glu Trp Ser Asp Pro Val Ile Phe Gln  
 210 215 220  
 Thr Gln Ser Glu Glu Leu Lys Glu Gly Trp Asn Pro His Leu Leu Leu  
 225 230 235 240  
 Leu Leu Leu Leu Val Ile Val Phe Ile Pro Ala Phe Trp Ser Leu Lys  
 245 250 255  
 Thr His Pro Leu Trp Arg Leu Trp Lys Lys Ile Trp Ala Val Pro Ser  
 260 265 270  
 Pro Glu Arg Phe Phe Met Pro Leu Tyr Lys Gly Cys Ser Gly Asp Phe  
 275 280 285  
 Lys Lys Trp Val Gly Ala Pro Phe Thr Gly Ser Ser Leu Glu Leu Gly  
 290 295 300  
 Pro Trp Ser Pro Glu Val Pro Ser Thr Leu Glu Val Tyr Ser Cys His  
 305 310 315 320  
 Pro Pro Arg Ser Pro Ala Lys Arg Leu Gln Leu Thr Glu Leu Gln Glu  
 325 330 335  
 Pro Ala Glu Leu Val Glu Ser Asp Gly Val Pro Lys Pro Ser Phe Trp  
 340 345 350  
 Pro Thr Ala Gln Asn Ser Gly Gly Ser Ala Tyr Ser Glu Glu Arg Asp  
 355 360 365  
 Arg Pro Tyr Gly Leu Val Ser Ile Asp Thr Val Thr Val Leu Asp Ala  
 370 375 380  
 Glu Gly Pro Cys Thr Trp Pro Cys Ser Cys Glu Asp Asp Gly Tyr Pro  
 385 390 395 400

Ala Leu Asp Leu Asp Ala Gly Leu Glu Pro Ser Pro Gly Leu Glu Asp  
                             405                            410                            415  
 Pro Leu Leu Asp Ala Gly Thr Thr Val Leu Ser Cys Gly Cys Val Ser  
                             420                            425                            430  
 Ala Gly Ser Pro Gly Leu Gly Gly Pro Leu Gly Ser Leu Leu Asp Arg  
                             435                            440                            445  
 Leu Lys Pro Pro Leu Ala Asp Gly Glu Asp Trp Ala Gly Gly Leu Pro  
                             450                            455                            460  
 Trp Gly Gly Arg Ser Pro Gly Gly Val Ser Glu Ser Glu Ala Gly Ser  
 465                            470                            475                            480  
 Pro Leu Ala Gly Leu Asp Met Asp Thr Phe Asp Ser Gly Phe Val Gly  
                             485                            490                            495  
 Ser Asp Cys Ser Ser Pro Val Glu Cys Asp Phe Thr Ser Pro Gly Asp  
                             500                            505                            510  
 Glu Gly Pro Pro Arg Ser Tyr Leu Arg Gln Trp Val Val Ile Pro Pro  
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17

&lt;210&gt; 24

&lt;211&gt; 18

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&lt;213&gt; Artificial Sequence

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18

&lt;210&gt; 25

&lt;211&gt; 33

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33

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